OLIGONUCLEOTIDE MODULATION OF CELL ADHESION

INTRODUCTION

This application is a continuation-in-part of application Serial No. 09/982,262, filed October 18, 2001, which is a continuation-in-part of application Serial No 09/659,288, filed September 12, 2000 (abandoned), which is a continuation of application Serial No. 09/128,496, filed August 3, 1998 (U.S. Patent No. 6,169,079), which is a continuation of application Serial No. 08/440,740, filed May 12, 1995 (U.S., Patent No. 5,843,738), which is a continuation-in-part of application Serial No. 08/063,167 filed May 17, 1993 (U.S. Patent No. 5,514,788), which is a continuation of application Serial No. 07/969,151 filed February 10, 1993 (abandoned), which is a continuation-in-part of application Serial No. 08/007,997 filed January 21, 1993 (U.S. Patent 5,591,623). The entire contents of these applications and patents is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to diagnostics, research reagents and therapies for disease states which respond to modulation of the synthesis or metabolism of cell adhesion molecules. In particular, this invention relates to antisense oligonucleotide interactions with certain messenger ribonucleic acids (mRNAs) or DNAs involved in the synthesis of proteins that regulate adhesion of white blood cells to other white blood cells and to other cell types. Antisense oligonucleotides designed to hybridize to the mRNA encoding intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1, also known as E-selectin), and vascular cell adhesion molecule-1 (VCAM-1) are provided. These oligonucleotides have been found to lead to the modulation of the activity of the RNA or DNA, and thus to the modulation of the synthesis and

metabolism of specific cell adhesion molecules. Palliation and therapeutic effect result.

BACKGROUND OF THE INVENTION

Inflammation is a localized protective response elicited by tissues in response to injury, infection, or tissue destruction resulting in the destruction of the infectious or injurious agent and isolation of the injured tissue. A typical inflammatory response proceeds as follows: recognition of an antigen as foreign or recognition of tissue damage, synthesis and release of soluble inflammatory mediators, recruitment of inflammatory cells to the site of infection or tissue damage, destruction and removal of the invading organism or damaged tissue, and deactivation of the system once the invading organism or damage has been resolved. In many human diseases with an inflammatory component, the normal, homeostatic mechanisms which attenuate the inflammatory responses are defective, resulting in damage and destruction of normal tissue.

Cell-cell interactions are involved in the activation of the immune response at each of the stages described above. One of the earliest detectable events in a normal inflammatory response is adhesion of leukocytes to the vascular endothelium, followed by migration of leukocytes out of the vasculature to the site of infection or injury. The adhesion of these leukocytes, or white blood cells, to vascular endothelium is an obligate step in the migration out of the vasculature. Harlan, J.M., *Blood* **1985**, 65, 513-525. In general, the first inflammatory cells to appear at the site of inflammation are neutrophils followed by monocytes, and lymphocytes. Cell-cell interactions are also critical for propagation of both B-lymphocytes and T-lymphocytes resulting in enhanced humoral and cellular immune responses, respectively.

The adhesion of white blood cells to vascular endothelium and other cell types is mediated by interactions between specific proteins, termed "adhesion molecules," located on the plasma

membrane of both white blood cells and vascular endothelium. The interaction between adhesion molecules is similar to classical receptor ligand interactions with the exception that the ligand is fixed to the surface of a cell instead of being soluble. The identification of patients with a genetic defect in leukocyte adhesion has enabled investigators to identify a family of proteins responsible for adherence of white blood cells. Leukocyte adhesion deficiency (LAD) is a rare autosomal trait characterized by recurrent bacterial infections and impaired pus formation and wound healing. The defect was shown to occur in the common B-subunit of three heterodimeric glycoproteins, Mac-1, LFA-1, and p150,95, normally expressed on the outer cell membrane of white blood cells. Anderson and Springer, *Ann. Rev. Med.* **1987**, 38, 175-194. Patients suffering from LAD exhibit a defect in a wide spectrum of adherence-dependent functions of granulocytes, monocytes, and lymphocytes. Three ligands for LFA-1 have been identified, intercellular adhesion molecules 1, 2 and 3 (ICAM-1, ICAM-2 and ICAM-3). Both Mac-1 and p150,95 bind complement fragment C3bi and perhaps other unidentified ligands. Mac-1 also binds ICAM-1.

Other adhesion molecules have been identified which are involved in the adherence of white blood cells to vascular endothelium and subsequent migration out of the vasculature. These include endothelial leukocyte adhesion molecule-1 (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1) and granule membrane protein-140 (GMP-140) and their respective receptors. The adherence of white blood cells to vascular endothelium appears to be mediated in part if not *in toto* by the five cell adhesion molecules ICAM-1, ICAM-2, ELAM-1, VCAM-1 and GMP-140. Dustin and Springer, *J. Cell Biol.* **1987**, 107, 321-331. Expression on the cell surface of ICAM-1, ELAM-1, VCAM-1 and GMP-140 adhesion molecules is induced by inflammatory stimuli. In contrast, expression of ICAM-2 appears to be constitutive and not sensitive to induction by cytokines. In general, GMP-140 is induced by autocoids such as histamine, leukotriene B_4, platelet activating

factor, and thrombin. Maximal expression on endothelial cells is observed 30 minutes to 1 hour after stimulation, and returns to baseline within 2 to 3 hours. The expression of ELAM-1 and VCAM-1 on endothelial cells is induced by cytokines such as interleukin-1ß and tumor necrosis factor, but not gamma-interferon. Maximal expression of ELAM-1 on the surface of endothelial cells occurs 4 to 6 hours after stimulation, and returns to baseline by 16 hours. ELAM-1 expression is dependent on new mRNA and protein synthesis. Elevated VCAM-1 expression is detectable 2 hours following treatment with tumor necrosis factor, is maximal 8 hours following stimulation, and remains elevated for at least 48 hours following stimulation. Rice and Bevilacqua, *Science* **1989**, 246, 1303-1306. ICAM-1 expression on endothelial cells is induced by cytokines interleukin-1 tumor necrosis factor and gamma-interferon. Maximal expression of ICAM-1 follows that of ELAM-1 occurring 8 to 10 hours after stimulation and remains elevated for at least 48 hours.

GMP-140 and ELAM-1 are primarily involved in the adhesion of neutrophils to vascular endothelial cells. VCAM-1 primarily binds T and B lymphocytes. In addition, VCAM-1 may play a role in the metastasis of melanoma, and possibly other cancers. ICAM-1 plays a role in adhesion of neutrophils to vascular endothelium, as well as adhesion of monocytes and lymphocytes to vascular endothelium, tissue fibroblasts and epidermal keratinocytes. ICAM-1 also plays a role in T-cell recognition of antigen presenting cell, lysis of target cells by natural killer cells, lymphocyte activation and proliferation, and maturation of T cells in the thymus. In addition, recent data have demonstrated that ICAM-1 is the cellular receptor for the major serotype of rhinovirus, which account for greater than 50% of common colds. Staunton et al., *Cell* **1989**, 56, 849-853; Greve et al., *Cell* **1989**, 56, 839-847.

Expression of ICAM-1 has been associated with a variety of inflammatory skin disorders such as allergic contact dermatitis, fixed drug eruption, lichen planus, and psoriasis; Ho et al., *J. Am. Acad. Dermatol.* **1990**, 22, 64-68; Griffiths and Nickoloff, *Am.*

J. Pathology **1989**, 135, 1045-1053; Lisby et al., *Br. J. Dermatol.* **1989**,120, 479-484; Shiohara et al., *Arch. Dermatol.* **1989**, 125, 1371-1376. In addition, ICAM-1 expression has been detected in the synovium of patients with rheumatoid arthritis; Hale et al., *Arth. Rheum.* **1989**, 32, 22-30, pancreatic B-cells in diabetes; Campbell et al., *Proc. Natl. Acad. Sci. U.S.A.* **1989**, 86, 4282-4286; thyroid follicular cells in patients with Graves' disease; Weetman et al., *J. Endocrinol.* **1989**, 122, 185-191; and with renal and liver allograft rejection; Faull and Russ, *Transplantation* **1989**, 48, 226-230; Adams et al., *Lancet* **1989**, 1122-1125. ICAM-1 is also expressed on corneal endothelial cells and is induced on corneal endothelial cells in response to inflammatory stimuli.

It is has been hoped that inhibitors of ICAM-1, VCAM-1 and ELAM-1 expression would provide a novel therapeutic class of anti-inflammatory agents with activity towards a variety of inflammatory diseases or diseases with an inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, various dermatological conditions, and psoriasis. In addition, inhibitors of ICAM-1, VCAM-1, and ELAM-1 may also be effective in the treatment of colds due to rhinovirus infection, AIDS, Kaposi's sarcoma and some cancers and their metastasis. To date, there are no known therapeutic agents which effectively prevent the expression of the cellular adhesion molecules ELAM-1, VCAM-1 and ICAM-1. The use of neutralizing monoclonal antibodies against ICAM-1 in animal models provide evidence that such inhibitors if identified would have therapeutic benefit for asthma; Wegner et al., *Science* **1990**, 247, 456-459, renal allografts; Cosimi et al., *J. Immunol.* **1990**, 144, 4604-4612, and cardiac allografts; Isobe et al., *Science* **1992**, 255, 1125-1127. The use of a soluble form of ICAM-1 molecule was also effective in preventing rhinovirus infection of cells in culture. Marlin et al., *Nature* **1990**, 344, 70-72.

Current agents which affect intercellular adhesion molecules include synthetic peptides, monoclonal antibodies, and soluble forms of the adhesion molecules. To date, synthetic peptides

which block the interactions with VCAM-1 or ELAM-1 have not been identified. Monoclonal antibodies may prove to be useful for the treatment of acute inflammatory response due to expression of ICAM-1, VCAM-1 and ELAM-1. However, with chronic treatment, the host animal develops antibodies against the monoclonal antibodies thereby limiting their usefulness. In addition, monoclonal antibodies are large proteins which may have difficulty in gaining access to the inflammatory site. Soluble forms of the cell adhesion molecules suffer from many of the same limitations as monoclonal antibodies in addition to the expense of their production and their low binding affinity. Thus, there is a long felt need for molecules which effectively inhibit intercellular adhesion molecules. Antisense oligonucleotides avoid many of the pitfalls of current agents used to block the effects of ICAM-1, VCAM-1 and ELAM-1.

PCT/US90/02357 (Hession et al.) discloses DNA sequences encoding Endothelial Adhesion Molecules (ELAMs), including ELAM-1 and VCAM-1 and VCAM-1b. A number of uses for these DNA sequences are provided, including (1) production of monoclonal antibody preparations that are reactive for these molecules which may be used as therapeutic agents to inhibit leukocyte binding to endothelial cells; (2) production of ELAM peptides to bind to the ELAM ligand on leukocytes which, in turn, may bind to ELAM on endothelial cells, inhibiting leukocyte binding to endothelial cells; (3) use of molecules binding to ELAMS (such as anti-ELAM antibodies, or markers such as the ligand or fragments of it) to detect inflammation; (4) use of ELAM and ELAM ligand DNA sequences to produce nucleic acid molecules that intervene in ELAM or ELAM ligand expression at the translational level using antisense nucleic acid and ribozymes to block translation of a specific MRNA either by masking MRNA with antisense nucleic acid or cleaving it with a ribozyme. It is disclosed that coding regions are the targets of choice. For VCAM-1, AUG is believed to be most likely; a 15-mer hybridizing to the AUG site is specifically disclosed in Example 17.

In the United States, 40,000 corneal transplants are performed per year. Human corneal allograft rejection is a major problem in corneal clinical practice. To date, no totally reliable and reproducible medication regimen provides assurance that allograft rejection will not occur in high risk patients, including those with corneal neovascularization and previous rejections. Corneal transplants require months of meticulous follow-up care, and significantly restrict the physical activity of recipients. In addition, corneal transplantation often necessitates general anesthesia and is very expensive. Therefore, allograft rejection presents significant personal, economic and anesthetic risks to patients. Thus, there is a need for compositions and methods which will prevent corneal allograft rejection.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is the mRNA sequence of human intercellular adhesion molecule-1 (ICAM-1).

FIGURE 2 is the mRNA sequence of human endothelial leukocyte adhesion molecule-1 (ELAM-1).

FIGURE 3 is the mRNA sequence of human vascular cell adhesion molecule-1 (VCAM-1).

FIGURE 4 is a graphical representation of the induction of ICAM-1 expression on the cell surface of various human cell lines by interleukin-1 and tumor necrosis factor.

FIGURE 5 is a graphical representation of the effects of selected antisense oligonucleotides on ICAM-1 expression on human umbilical vein endothelial cells.

FIGURE 6A and 6B are a graphical representation of the effects of an antisense oligonucleotide on the expression of ICAM-1 in human umbilical vein endothelial cells stimulated with tumor necrosis factor and interleukin-1.

FIGURE 7 is a graphical representation of the effect of antisense oligonucleotides on ICAM-1 mediated adhesion of DMSO differentiated HL-60 cells to control and tumor necrosis factor

treated human umbilical vein endothelial cells.

FIGURE 8 is a graphical representation of the effects of selected antisense oligonucleotides on ICAM-1 expression in A549 human lung carcinoma cells.

FIGURE 9 is a graphical representation of the effects of selected antisense oligonucleotides on ICAM-1 expression in primary human keratinocytes.

FIGURE 10 is a graphical representation of the relationship between oligonucleotide chain length, Tm and effect on inhibition of ICAM-1 expression.

FIGURE 11 is a graphical representation of the effect of selected antisense oligonucleotides on ICAM-1 mediated adhesion of DMSO differentiated HL-60 cells to control and tumor necrosis factor treated human umbilical vein endothelial cells.

FIGURE 12 is a graphical representation of the effects of selected antisense oligonucleotides on ELAM-1 expression on tumor necrosis factor-treated human umbilical vein endothelial cells.

FIGURE 13 is a graphical representation of the human ELAM-1 mRNA showing target sites of antisense oligonucleotides.

FIGURE 14 is a graphical representation of the human VCAM-1 mRNA showing target sites of antisense oligonucleotides.

FIGURE 15 is a line graph showing inhibition of ICAM-1 expression in C8161 human melanoma cells following treatment with antisense oligonucleotides complementary to ICAM-1.

FIGURE 16 is a bar graph showing the effect of ISIS 3082 on dextran sulfate (DSS)-induced inflammatory bowel disease.

FIGURE 17 is a graph showing the effects of ICAM-1 antisense oligonucleotides (ISIS 13315 and 17481) on airway resistance in an ovalbumin-induced mouse asthma model after intratracheal oligonucleotide administration. Penh is a measure of airway resistance. Naïve mice were not sensitized with ovalbumin.

FIGURE 18 is a graph showing the effects of ICAM-1 antisense oligonucleotides (ISIS 13315 and 17481) on the number of eosonophils in bronchiolar lavage (BAL) fluid in an ovalbumin-induced mouse asthma model after intratracheal oligonucleotide

administration.

FIGURE 19 is a graph showing the effects of ICAM-1 antisense oligonucleotides (ISIS 13315 and 17481) on the number of neutrophils in bronchiolar lavage (BAL) fluid in an ovalbumin-induced mouse asthma model after intratracheal oligonucleotide administration.

FIGURE 20 is a graph showing the effect of pretreatment with an ICAM-1 antisense oligonucleotide (ISIS 10984) on pulmonary methacholine responsiveness in ascaris sensitive cynomolgus monkeys.

SUMMARY OF THE INVENTION

In accordance with the present invention, oligonucleotides are provided which specifically hybridize with nucleic acids encoding intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1). The oligonucleotides are designed to bind either directly to mRNA or to a selected DNA portion forming a triple stranded structure, thereby modulating the amount of mRNA made from the gene. This relationship is commonly denoted as "antisense."

Oligonucleotides are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed for research use. This specificity and sensitivity is also harnessed by those of skill in the art for diagnostic uses.

It is preferred to target specific genes for antisense attack. "Targeting" an oligonucleotide to the associated ribonucleotides, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic

acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a cellular gene associated with a particular disease state. The targeting process also includes determination of a site or sites within this region for the oligonucleotide interaction to occur such that the desired effect, either detection of or modulation of expression of the protein will result. Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

"Hybridization", in the context of this invention, means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

It is understood in the art that the sequence of the oligomeric compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligomeric compound may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). It is preferred that the oligomeric compounds of the present invention comprise at least 70% sequence complementarity to a target region within the target nucleic acid, more preferably that they comprise 90% sequence complementarity and even more preferably comprise 95% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an oligomeric compound in which 18 of 20 nucleobases of the oligomeric compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an oligomeric compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an oligomeric compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656).

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which an oligomeric compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will vary with different circumstances and in the context of this invention;

"stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

It has been discovered that the genes coding for ICAM-1, VCAM-1 and ELAM-1 are particularly useful for this approach. Inhibition of ICAM-1, VCAM-1 and ELAM-1 expression is expected to be useful for the treatment of inflammatory diseases, diseases with an inflammatory component, allograft rejection, psoriasis and other skin diseases, inflammatory bowel disease, cancers and their metastasis, and viral infections.

Methods of modulating cell adhesion comprising contacting the animal with an oligonucleotide hybridizable with nucleic acids encoding a protein capable of modulating cell adhesion are provided. Oligonucleotides hybridizable with an RNA or DNA encoding ICAM-1, VCAM-1 and ELAM-1 are preferred.

The present invention is also useful in diagnostics and in research. Since the oligonucleotides of this invention hybridize to ICAM-1, ELAM-1 or VCAM-1, sandwich and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of an oligonucleotide with one of these intercellular adhesion molecules present in a sample suspected of containing it can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection system. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with a detectably labeled oligonucleotide of the present invention under conditions selected to permit hybridization and measuring such hybridization by detection of the label.

For example, radiolabeled oligonucleotides can be prepared by ^{32}P labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 10.59. Radiolabeled oligonucleotides are then contacted with tissue or

cell samples suspected of containing an intercellular adhesion molecule and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide (which in turn indicates the presence of an intercellular adhesion molecule) and can be quantitated using a scintillation counter or other routine means. Expression of these proteins can then be detected.

Radiolabeled oligonucleotides of the present invention can also be used to perform autoradiography of tissues to determine the localization, distribution and quantitation of intercellular adhesion molecules for research, diagnostic or therapeutic purposes. In such studies, tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to routine autoradiography procedures. The emulsion, when developed, yields an image of silver grains over the regions expressing a intercellular adhesion molecule. Quantitation of the silver grains permits expression of these molecules to be detected and permits targeting of oligonucleotides to these areas.

Analogous assays for fluorescent detection of expression of intercellular adhesion molecules can be developed using oligonucleotides of the present invention which are conjugated with fluorescein or other fluorescent tag instead of radiolabeling. Such conjugations are routinely accomplished during solid phase synthesis using fluorescently labeled amidites or CPG (e.g., fluorescein-labeled amidites and CPG available from Glen Research, Sterling VA).

Each of these assay formats is known in the art. One of skill could easily adapt these known assays for detection of expression of intercellular adhesion molecules in accordance with the teachings of the invention providing a novel and useful means to detect expression of these molecules. Antisense oligonucleotide inhibition of the expression of intercellular adhesion molecules *in vitro* is useful as a means to determine a proper course of therapeutic treatment. For example, before a

patient is treated with an oligonucleotide composition of the present invention, cells, tissues or a bodily fluid from the patient can be treated with the oligonucleotide and inhibition of expression of intercellular adhesion molecules can be assayed. Effective *in vitro* inhibition of the expression of molecules in the sample indicates that the expression will also be modulated *in vivo* by this treatment.

Kits for detecting the presence or absence of intercellular adhesion molecules may also be prepared. Such kits include an oligonucleotide targeted to ICAM-1, ELAM-1 or VCAM-1.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations, and in other methodologies which may be appreciated by persons of ordinary skill in the art.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Antisense oligonucleotides hold great promise as therapeutic agents for the treatment of many human diseases. Oligonucleotides specifically bind to the complementary sequence of either pre-mRNA or mature mRNA, as defined by Watson-Crick base pairing, inhibiting the flow of genetic information from DNA to protein. The properties of antisense oligonucleotides, which make them specific for their target sequence, also make them extraordinarily versatile. Because antisense oligonucleotides are long chains of four monomeric units they may be readily synthesized for any target RNA sequence. Numerous recent studies have documented the utility of antisense oligonucleotides as biochemical tools for studying target proteins. Rothenberg et al., *J. Natl. Cancer Inst.* **1989**, 81, 1539-1544; Zon, G. *Pharmaceutical Res.* **1988**, 5, 539-549). Because of recent advances in synthesis of nuclease resistant oligonucleotides, which exhibit enhanced cell uptake, it is now possible to consider the use of antisense oligonucleotides as a novel form of therapeutics.

Antisense oligonucleotides offer an ideal solution to the

problems encountered in prior art approaches. They can be designed to selectively inhibit a given isoenzyme, they inhibit the production of the enzyme, and they avoid non-specific mechanisms such as free radical scavenging or binding to multiple receptors. A complete understanding of enzyme mechanisms or receptor-ligand interactions is not needed to design specific inhibitors.

DESCRIPTION OF TARGETS

The acute infiltration of neutrophils into the site of inflammation appears to be due to increased expression of GMP-140, ELAM-1 and ICAM-1 on the surface of endothelial cells. The appearance of lymphocytes and monocytes during the later stages of an inflammatory reaction appear to be mediated by VCAM-1 and ICAM-1. ELAM-1 and GMP-140 are transiently expressed on vascular endothelial cells, while VCAM-1 and ICAM-1 are chronically expressed.

Human ICAM-1 is encoded by a 3.3-kb mRNA resulting in the synthesis of a 55,219 dalton protein (Figure 1). ICAM-1 is heavily glycosylated through N-linked glycosylation sites. The mature protein has an apparent molecular mass of 90 kDa as determined by SDS-polyacrylamide gel electrophoresis. Staunton et al., *Cell* **1988**, 52, 925-933. ICAM-1 is a member of the immunoglobulin supergene family, containing 5 immunoglobulin-like domains at the amino terminus, followed by a transmembrane domain and a cytoplasmic domain. The primary binding site for LFA-1 and rhinovirus are found in the first immunoglobulin-like domain. However, the binding sites appear to be distinct. Staunton et al., *Cell* **1990**, 61, 243-354. Recent electron micrographic studies demonstrate that ICAM-1 is a bent rod 18.7 nm in length and 2 to 3 nm in diameter. Staunton et al., *Cell* **1990**, 61, 243-254.

ICAM-1 exhibits a broad tissue and cell distribution, and may be found on white blood cells, endothelial cells, fibroblast, keratinocytes and other epithelial cells. The expression of ICAM-1 can be regulated on vascular endothelial cells, fibroblasts,

keratinocytes, astrocytes and several cell lines by treatment with bacterial lipopolysaccharide and cytokines such as interleukin-1, tumor necrosis factor, gamma-interferon, and lymphotoxin. *See, e.g.*, Frohman et al., *J. Neuroimmunol.* **1989**, 23, 117-124. The molecular mechanism for increased expression of ICAM-1 following cytokine treatment has not been determined.

ELAM-1 is a 115-kDa membrane glycoprotein (Figure 2) which is a member of the selectin family of membrane glycoproteins. Bevilacqua et al., *Science* **1989**, 243, 1160-1165. The amino terminal region of ELAM-1 contains sequences with homologies to members of lectin-like proteins, followed by a domain similar to epidermal growth factor, followed by six tandem 60-amino acid repeats similar to those found in complement receptors 1 and 2. These features are also shared by GMP-140 and MEL-14 antigen, a lymphocyte homing antigen. ELAM-1 is encoded for by a 3.9-kb mRNA. The 3'-untranslated region of ELAM-1 mRNA contains several sequence motifs ATTTA which are responsible for the rapid turnover of cellular mRNA consistent with the transient nature of ELAM-1 expression.

ELAM-1 exhibits a limited cellular distribution in that it has only been identified on vascular endothelial cells. Like ICAM-1, ELAM-1 is inducible by a number of cytokines including tumor necrosis factor, interleukin-1 and lymphotoxin and bacterial lipopolysaccharide. In contrast to ICAM-1, ELAM-1 is not induced by gamma-interferon. Bevilacqua et al., *Proc. Natl. Acad. Sci. USA* **1987**, 84, 9238-9242; Wellicome et al., *J. Immunol.* **1990**, 144, 2558-2565. The kinetics of ELAM-1 mRNA induction and disappearance in human umbilical vein endothelial cells precedes the appearance and disappearance of ELAM-1 on the cell surface. As with ICAM-1, the molecular mechanism for ELAM-1 induction is not known.

VCAM-1 is a 110-kDa membrane glycoprotein encoded by a 3.2-kb mRNA (Figure 3). VCAM-1 appears to be encoded by a single-copy gene which can undergo alternative splicing to yield products with either six or seven immunoglobulin domains. Osborn et al., *Cell*

1989, 59, 1203-1211. The receptor for VCAM-1 is proposed to be CD29 (VLA-4) as demonstrated by the ability of monoclonal antibodies to CD29 to block adherence of Ramos cells to VCAM-1. VCAM-1 is expressed primarily on vascular endothelial cells. Like ICAM-1 and ELAM-1, expression of VCAM-1 on vascular endothelium is regulated by treatment with cytokines. Rice and Bevilacqua, *Science* **1989**, 246, 1303-1306; Rice et al., *J. Exp. Med.* **1990**, 171, 1369-1374. Increased expression appears to be due to induction of the mRNA.

For therapeutics, an animal suspected of having a disease which can be treated by decreasing the expression of ICAM-1, VCAM-1 and ELAM-1 is treated by administering oligonucleotides in accordance with this invention. Oligonucleotides may be formulated in a pharmaceutical composition, which may include carriers, thickeners, diluents, buffers, preservatives, surface active agents, liposomes or lipid formulations and the like, in addition to the oligonucleotide. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like, in addition to oligonucleotide.

The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection.

In another embodiment, the administration is pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer, dry powder inhaler, or metered dose inhaler; intratracheal or intranasal.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be

necessary or desirable. Coated condoms or gloves may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for oral administration also include pulsatile delivery compositions and bioadhesive composition as described in copending U.S. Patent Application Serial Nos. 09/944,493, filed August 22, 2001, and 09/935,316, filed August 22, 2001, the entire disclosures of which are incorporated herein by reference.

Formulations for parenteral administration may include sterile aqueous solutions, which may also contain buffers, liposomes, diluents and other suitable additives.

Dosing is dependent on severity and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

The present invention employs oligonucleotides for use in antisense inhibition of the function of RNA and DNA corresponding to proteins capable of modulating inflammatory cell adhesion. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-

stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, **1995**, 81, 611-620).Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine (or uridine if RNA), guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three alternate nucleotides. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of ICAM-1, VCAM-1 or ELAM-1.

Oligomer and Monomer Modifications

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside linkage or in conjunction with the sugar ring the backbone of the oligonucleotide. The normal internucleoside linkage that makes up the backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages

Specific examples of preferred antisense oligomeric compounds useful in this invention include oligonucleotides containing modified e.g. non-naturally occurring internucleoside linkages. As defined in this specification, oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom and internucleoside linkages that do not have a phosphorus atom. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly

interfere with RNAi activity. Based on this observation, it is suggested that certain preferred oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is the phosphorothioate internucleoside linkage.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular $-CH_2-NH-O-CH_2-$, $-CH_2-$

$N(CH_3)$-O-CH_2- [known as a methylene (methylimino) or MMI backbone], -CH_2-O-$N(CH_3)$-CH_2-, -CH_2-$N(CH_3)$-$N(CH_3)$-CH_2- and -O-$N(CH_3)$-CH_2-CH_2- [wherein the native phosphodiester internucleotide linkage is represented as -O-P(=O)(OH)-O-CH_2-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

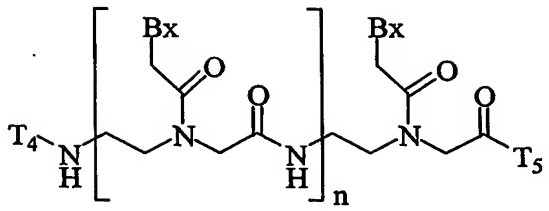
Oligomer Mimetics

Another preferred group of oligomeric compounds amenable to the present invention includes oligonucleotide mimetics. The term mimetic as it is applied to oligonucleotides is intended to

include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. The heterocyclic base moiety or a modified heterocyclic base moiety is maintained for hybridization with an appropriate target nucleic acid. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA oligomeric compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA oligomeric compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA oligomeric compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

One oligonucleotide mimetic that has been reported to have excellent hybridization properties is peptide nucleic acids (PNA). The backbone in PNA compounds is two or more linked aminoethylglycine units which gives PNA an amide containing backbone. The heterocyclic base moieties are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein

Bx is a heterocyclic base moiety;

T_4 is hydrogen, an amino protecting group, $-C(O)R_5$, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group, a reporter group, a conjugate group, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

T_5 is $-OH$, $-N(Z_1)Z_2$, R_5, D or L α-amino acid linked via the α-amino group or optionally through the ω-amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

Z_1 is hydrogen, C_1-C_6 alkyl, or an amino protecting group;

Z_2 is hydrogen, C_1-C_6 alkyl, an amino protecting group, $-C(=O)-(CH_2)_n-J-Z_3$, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

Z_3 is hydrogen, an amino protecting group, $-C_1$-C_6 alkyl, $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$;

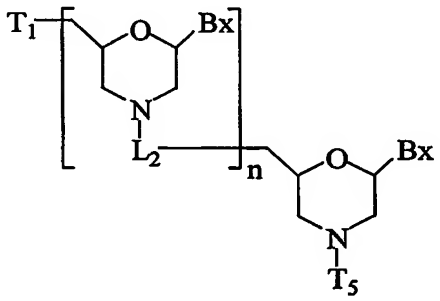
each J is O, S or NH;

R_5 is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41(14)*, 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The basic formula is shown below:



wherein

T_1 is hydroxyl or a protected hydroxyl;

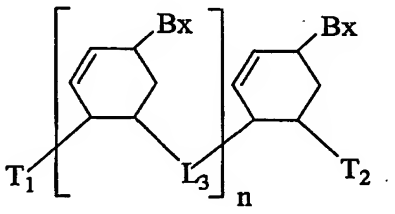
T_5 is hydrogen or a phosphate or phosphate derivative;

L_2 is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al.*, *J. Am. Chem. Soc.*, **2000**, *122*, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate *E. coli* RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



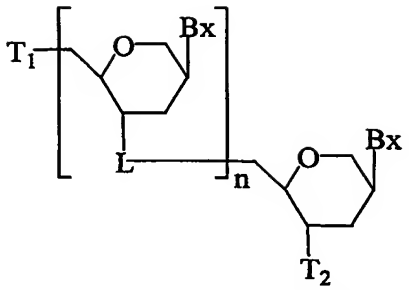
wherein

each Bx is a heterocyclic base moiety;

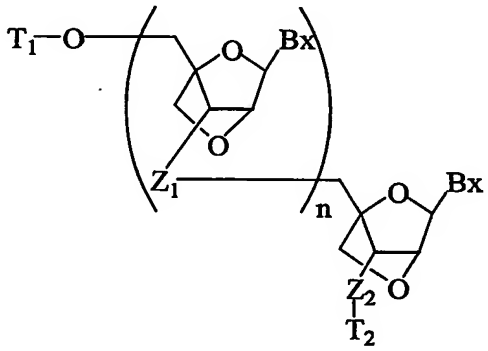
T_1 is hydroxyl or a protected hydroxyl; and

T2 is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 1563-1566) and would have the general formula:



A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10 C), stability towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:



The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol.

Recognit., 2000, 13, 44-53). These conformations are associated with improved stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points (Tm = +15/+11) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

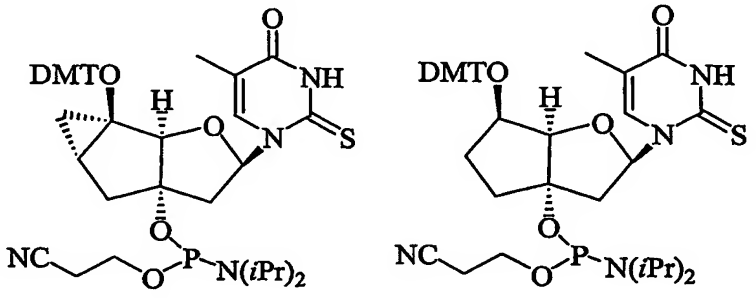
Novel types of LNA-oligomeric compounds, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs. Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily

in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor signaling in living rat brain and detection of reporter genes in *E.* coli. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

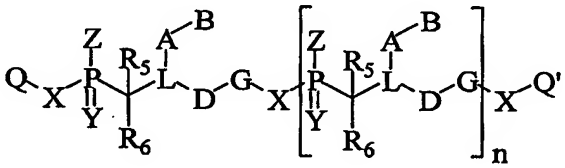
Further oligonucleotide mimetics have been prepared to include bicyclic and tricyclic nucleoside analogs having the formulas (amidite monomers shown):



(see Steffens *et al.*, *Helv. Chim. Acta*, **1997**, *80*, 2426-2439; Steffens *et al.*, *J. Am. Chem. Soc.*, **1999**, *121*, 3249-3255; and Renneberg *et al.*, *J. Am. Chem. Soc.*, **2002**, *124*, 5993-6002). These modified nucleoside analogs have been oligomerized using the phosphoramidite approach and the resulting oligomeric compounds containing tricyclic nucleoside analogs have shown increased thermal stabilities (Tm's) when hybridized to DNA, RNA and itself. Oligomeric compounds containing bicyclic nucleoside analogs have shown thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus group in a backbone the backbone. This class of olignucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.



Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

Modified sugars

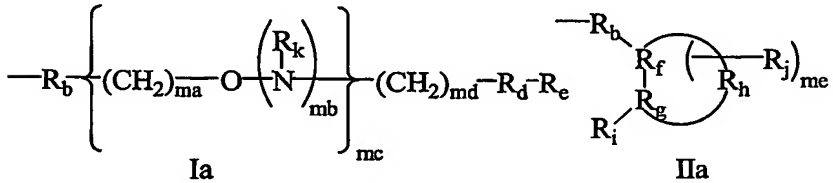
Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or

O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise a sugar substituent group selected from: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$.

Other preferred sugar substituent groups include methoxy (-O-CH_3), aminopropoxy (-$OCH_2CH_2CH_2NH_2$), allyl (-CH_2-CH=CH_2), -O-allyl (-O-CH_2-CH=CH_2) and fluoro (F). 2'-Sugar substituent groups may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligomeric compound, particularly the 3' position of the sugar on the 3' terminal nucleoside or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligomeric compounds may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080;

5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Further representative sugar substituent groups include groups of formula I_a or II_a:



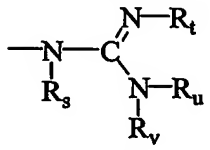
Ia IIa

wherein:

R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

R_e is C_1-C_{10} alkyl, $N(R_k)(R_m)$, $N(R_k)(R_n)$, $N=C(R_p)(R_q)$, $N=C(R_p)(R_r)$ or has formula III_a;



IIIa

R_p and R_q are each independently hydrogen or C_1-C_{10} alkyl;

R_r is $-R_x-R_y$;

each R_s, R_t, R_u and R_v is, independently, hydrogen, $C(O)R_w$, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_w is, independently, substituted or unsubstituted C_1-C_{10} alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_k is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

R_p is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH_3^+, $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or R_m and R_n, together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

R_i is OR_z, SR_z, or $N(R_z)_2$;

each R_z is, independently, H, C_1-C_8 alkyl, C_1-C_8 haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

R_f, R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ OR_k, halo, SR_k or CN;

m_a is 1 to about 10;

each mb is, independently, 0 or 1;
mc is 0 or an integer from 1 to 10;
md is an integer from 1 to 10;
me is from 0, 1 or 2; and
provided that when mc is 0, md is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Oligomeric compounds that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby incorporated by reference in its entirety.

Representative dimethylaminoethyloxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethyloxyethyl-Oligomeric compounds", filed August 6, 1999, hereby incorporated by reference in its entirety.

Modified Nucleobases/Naturally occurring nucleobases

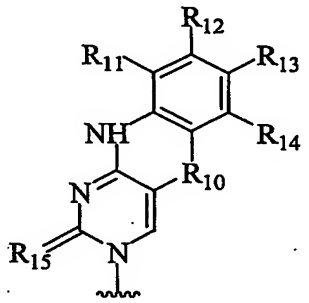
Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions. As used herein,

"unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex

stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

In one aspect of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:



Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one (R_{10} = O, R_{11} - R_{14}= H) [Kurchavov, *et al.*, *Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one (R_{10}= S, R_{11} - R_{14}= H), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. Am. Chem. Soc. 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one (R_{10} = O, R_{11} - R_{14} = F) [Wang, J.; Lin, K.-Y., Matteucci, M. Tetrahedron Lett. 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions(also see U.S.

Patent Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold (R_{10} = O, R_{11} = -O-$(CH_2)_2$-NH_2, R_{12-14}=H) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine ($dC5^{me}$), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater discrimination between the perfect match and mismatched sequences compared to $dC5^{me}$. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent

antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K-Y; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful as heterocyclcic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and Unites States Patent Application Serial number 09/996,292 filed November 28, 2001, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three alternate nucleotides. These oligonucleotides are then tested using the methods described

herein to determine their ability to inhibit expression of HCV mRNA and/or HCV replication.

Conjugates

A further preferred substitution that can be appended to the oligomeric compounds of the invention involves the linkage of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting oligomeric compounds. In one embodiment such modified oligomeric compounds are prepared by covalently attaching conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl

residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

The oligomeric compounds of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142;

5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric oligomeric compounds

It is not necessary for all positions in an oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric compounds or "chimeras," in the context of this invention, are oligomeric compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide

mimetics as described above. Such oligomeric compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

3'-endo modifications

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can incorporate synthetic modifications of the heterocyclic base, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric compound can be enhanced while maintaining the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement (e.g. trigger) of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The present invention provides oligomeric triggers of RNAi having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

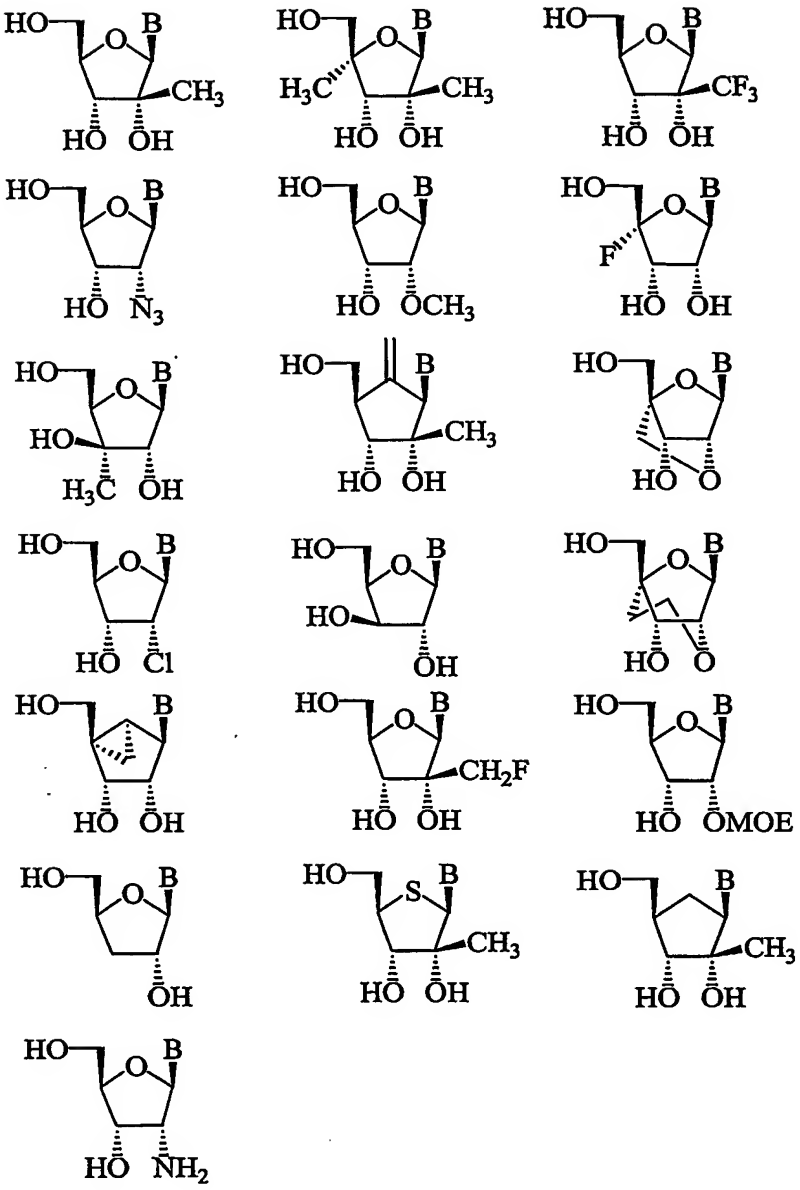
Scheme 1

C2'-endo/Southern C3'-endo/Northern

Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2, below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.) Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'deoxy-2'F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J. Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters

(2002), 12, 73-76.) Examples of modified nucleosides amenable to the present invention are shown below in Table I. These examples are meant to be representative and not exhaustive.

Table I



The preferred conformation of modified nucleosides and their oligomers can be estimated by various methods such as molecular dynamics calculations, nuclear magnetic resonance spectroscopy and CD measurements. Hence, modifications predicted to induce RNA

like conformations, A-form duplex geometry in an oligomeric context, are selected for use in the modified oligoncleotides of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known in the art (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press., and the examples section below.) Nucleosides known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B

In one aspect, the present invention is directed to oligonucleotides that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligonucleotide is selected having an effective length and sequence that is complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much cheaper and easier to synthesize and/or incorporate into an oligonucleotide. The selected sequence can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the 5' and 3'-termini as there are often advantageous modifications that can be made to one or more of the terminal nucleosides. The oligomeric compounds of the present invention include at least one 5'-modified phosphate group on a single strand or on at least one 5'-position of a double stranded sequence or sequences. Further modifications are also considered such as internucleoside linkages, conjugate groups, substitute sugars or bases, substitution of one or more nucleosides with nucleoside mimetics and any other modification that can enhance the selected sequence for its intended target. The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and "B Form" for DNA. The respective conformational

geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, **1970**, *47*, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (Tm's) than DNA:DNA duplexes (Sanger et al., Principles of Nucleic Acid Structure, 1984, Springer-Verlag; New York, NY.; Lesnik et al., Biochemistry, 1995, 34, 10807-10815; Conte et al., Nucleic Acids Res., 1997, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., Nucleic Acids Res., 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., Biochemistry, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) Principles of Nucleic Acid Structure, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, *et. al.*, *Nucleic Acids Research*, **1998**, *26*, 2473-2480, who pointed out that in considering the furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al.*, *Nucleic Acids Res.*, **1993**, *21*, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al.*, *Eur. J. Biochem.*, **1993**, *215*, 297-306; Fedoroff *et al.*, *J. Mol. Biol.*, **1993**, *233*, 509-523; Gonzalez *et al.*, *Biochemistry*, **1995**, *34*, 4969-

4982; Horton *et al.*, *J. Mol. Biol.*, **1996**, *264*, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

One routinely used method of modifying the sugar puckering is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependent on the nature of the substituent at the 2'-position. A number of different substituents have been studied to determine their sugar puckering effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH) versus deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form duplex. Data from UV hypochromicity, circular dichroism, and ^{1}H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number of effects on the stacking

conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-$OCH_2CH_2OCH_3$) side chain (Baker *et al.*, *J. Biol. Chem.*, **1997**, *272*, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-*O*-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995**, *78*, 486-504; Altmann *et al.*, *Chimia*, **1996**, *50*, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, *24*, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, *16*, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE modification displayed improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

Chemistries Defined

Unless otherwise defined herein, alkyl means C_1-C_{12}, preferably C_1-C_8, and more preferably C_1-C_6, straight or (where possible) branched chain aliphatic hydrocarbyl.

Unless otherwise defined herein, heteroalkyl means C_1-C_{12}, preferably C_1-C_8, and more preferably C_1-C_6, straight or (where possible) branched chain aliphatic hydrocarbyl containing at least one, and preferably about 1 to about 3, hetero atoms in the chain, including the terminal portion of the chain. Preferred heteroatoms include N, O and S. Unless otherwise defined herein, cycloalkyl means C_3-C_{12}, preferably C_3-C_8, and more preferably C_3-C_6, aliphatic hydrocarbyl ring.

Unless otherwise defined herein, alkenyl means C_2-C_{12}, preferably C_2-C_8, and more preferably C_2-C_6 alkenyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon double bond.

Unless otherwise defined herein, alkynyl means C_2-C_{12}, preferably C_2-C_8, and more preferably C_2-C_6 alkynyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon triple bond.

Unless otherwise defined herein, heterocycloalkyl means a ring moiety containing at least three ring members, at least one of which is carbon, and of which 1, 2 or three ring members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodinyl, tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrrazolyl, furanyl, pyranyl, and tetrahydroisothiazolyl.

Unless otherwise defined herein, aryl means any hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred

aryl rings include phenyl, napthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety, such as hetarylalkyl (hetaryl and alkyl), aralkyl (aryl and alkyl), etc., each of the sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is attached. Other electron withdrawing groups of note include those whose electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I.

The aforementioned optional substituents are, unless otherwise herein defined, suitable substituents depending upon desired properties. Included are halogens (Cl, Br, I), alkyl, alkenyl, and alkynyl moieties, NO_2, NH_3 (substituted and unsubstituted), acid moieties (e.g. $-CO_2H$, $-OSO_3H_2$, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc.

In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or sulfur of the 5'-phosphate.

Phosphate protecting groups include those described in US

Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

Affinity of an oligonucleotide for its target (in this case a nucleic acid encoding HCV RNA) is routinely determined by measuring the Tm of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the Tm, the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is modified to increase HCV RNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher Tm (i.e., higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance antisense oligonucleotide inhibition of HCV RNA function. RNAse H is a cellular endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of antisense inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred embodiment, the chimeric oligonucleotide is also modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel

electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. Oligonucleotides which contain at least one phosphorothioate modification are presently more preferred.

The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 80 nucleic acid base units. It is more preferred that such oligonucleotides comprise from about 12 to 50 nucleic acid base units, and still more preferred to have from about 15 to 30 nucleic acid base units. As will be appreciated, a nucleic acid base unit is a base-sugar combination suitably bound to an adjacent nucleic acid base unit through phosphodiester or other bonds.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; however, the actual synthesis of the oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA identified by the open reading frames (ORFs) of the DNA from which they are transcribed includes not only the information from the ORFs of the DNA, but also associated ribonucleotides which form regions known to such persons as the 5'-untranslated region, the 3'-untranslated region, and intervening sequence ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention, which are targeted wholly or in part to these

associated ribonucleotides as well as to the informational ribonucleotides. In preferred embodiments, the oligonucleotide is specifically hybridizable with a transcription initiation site, a translation initiation site, an intervening sequence and sequences in the 3'-untranslated region.

In accordance with this invention, the oligonucleotide is specifically hybridizable with portions of nucleic acids encoding a protein involved in the adhesion of white blood cells either to other white blood cells or other cell types. In preferred embodiments, said proteins are intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule-1. Oligonucleotides comprising the corresponding sequence, or part thereof, are useful in the invention. For example, Figure 1 is a human intercellular adhesion molecule-1 mRNA sequence. A preferred sequence segment, which may be useful in whole or in part, is:

5' 3'	SEQ ID NO:
TGGGAGCCATAGCGAGGC	1
GAGGAGCTCAGCGTCGACTG	2
GACACTCAATAAATAGCTGGT	3
GAGGCTGAGGTGGGAGGA	4
CGATGGGCAGTGGGAAAG	5
GGGCGCGTGATCCTTATAGC	6
CATAGCGAGGCTGAGGTTGC	7
CGGGGGCTGCTGGGAGCCAT	8
TCAGGGAGGCGTGGCTTGTG	13
CCTGTCCCGGGATAGGTTCA	14
TTGAGAAAGCTTTATTAACT	16
CCCCCACCACTTCCCCTCTC.	15

Figure 2 is a human endothelial leukocyte adhesion molecule-1 mRNA sequence. A preferred sequence segment, which may be useful in whole or in part, is:

5' 3'	SEQ ID NO:
CAATCATGACTTCAAGAGTTCT	28

TCACTGCTGCCTCTGTCTCAGG	73
TGATTCTTTTGAACTTAAAAGGA	74
TTAAAGGATGTAAGAAGGCT	75
CATAAGCACATTTATTGTC	76
TTTTGGGAAGCAGTTGTTCA	77
AACTGTGAAGCAATCATGACT	78
CCTTGAGTGGTGCATTCAACCT	79
AATGCTTGCTCACACAGGCATT	80.

Figure 3 is a human vascular cell adhesion molecule-1 mRNA sequence. A preferred sequence segment, which may be useful in whole or in part, is:

5' 3'	SEQ ID NO:
CCAGGCATTTTAAGTTGCTGT	40
CCTGAAGCCAGTGAGGCCCG	41
GATGAGAAAATAGTGGAACCA	42
CTGAGCAAGATATCTAGAT	43
CTACACTTTTGATTTCTGT	44
TTGAACATATCAAGCATTAGCT	45
TTTACATATGTACAAATTATGT	46
AATTATCACTTTACTATACAAA	47
AGGGCTGACCAAGACGGTTGT	48.

While the illustrated sequences are believed to be accurate, the present invention is directed to the correct sequences, should errors be found. Oligonucleotides useful in the invention comprise one of these sequences, or part thereof. Thus, it is preferred to employ any of these oligonucleotides as set forth above or any of the similar oligonucleotides which persons of ordinary skill in the art can prepare from knowledge of the preferred antisense targets for the modulation of the synthesis of inflammatory cell adhesion molecules.

Several preferred embodiments of this invention are exemplified in accordance with the following nonlimiting examples. The target mRNA species for modulation relates to intercellular

adhesion molecule-1, endothelial leukocyte adhesion molecule-1, and vascular cell adhesion molecule-1. Persons of ordinary skill in the art will appreciate that the present invention is not so limited, however, and that it is generally applicable. The inhibition or modulation of production of the ICAM-1 and/or ELAM-1 and/or VCAM-1 are expected to have significant therapeutic benefits in the treatment of disease. In order to assess the effectiveness of the compositions, an assay or series of assays is performed.

One type of disorder suitable for treatment with the oligonucleotides of the present invention are in inflammatory ophthalmic disorders including redness and inflammation caused by allergens and allergic reactions. The oligonucleotides can also be used as an adjuvant to antibiotic treatment of conjunctivitis. In a preferred embodiment, the oligonucleotides are used to preserve corneal explants *ex vivo* and to prevent corneal allograft rejection. These oligonucleotides may be placed in solution and administered as eyedrops for topical treatment of the allograft. The solution is suitable for use as a storage medium for corneal explants, and is administered in eye drop form following corneal transplant to prevent corneal allograft rejection.

The following examples are provided for illustrative purposes only and are not intended to limit the invention.

EXAMPLES

Example 1

Expression of ICAM-1, VCAM-1 and ELAM-1 on the surface of cells can be quantitated using specific monoclonal antibodies in an ELISA. Cells are grown to confluence in 96 well microtiter plates. The cells are stimulated with either interleukin-1 or tumor necrosis factor for 4 to 8 hours to quantitate ELAM-1 and 8 to 24 hours to quantitate ICAM-1 and VCAM-1. Following the appropriate incubation time with the cytokine, the cells are gently washed three times with a buffered isotonic solution containing calcium and magnesium such as Dulbecco's phosphate

buffered saline (D-PBS). The cells are then directly fixed on the microtiter plate with 1 to 2% paraformaldehyde diluted in D-PBS for 20 minutes at 25°C. The cells are washed again with D-PBS three times. Nonspecific binding sites on the microtiter plate are blocked with 2% bovine serum albumin in D-PBS for 1 hour at 37°C. Cells are incubated with the appropriate monoclonal antibody diluted in blocking solution for 1 hour at 37°C. Unbound antibody is removed by washing the cells three times with D-PBS. Antibody bound to the cells is detected by incubation with a 1:1000 dilution of biotinylated goat anti-mouse IgG (Bethesda Research Laboratories, Gaithersburg, MD) in blocking solution for 1 hour at 37°C. Cells are washed three times with D-PBS and then incubated with a 1:1000 dilution of streptavidin conjugated to ß-galactosidase (Bethesda Research Laboratories) for 1 hour at 37°C. The cells are washed three times with D-PBS for 5 minutes each. The amount of ß-galactosidase bound to the specific monoclonal antibody is determined by developing the plate in a solution of 3.3 mM chlorophenolred-ß-D-galactopyranoside, 50 mM sodium phosphate, 1.5 mM $MgCl_2$; pH=7.2 for 2 to 15 minutes at 37°C. The concentration of the product is determined by measuring the absorbance at 575 nm in an ELISA microtiter plate reader.

An example of the induction of ICAM-1 observed following stimulation with either interleukin-1ß or tumor necrosis factor α in several human cell lines is shown in Figure 4. Cells were stimulated with increasing concentrations of interleukin-1 or tumor necrosis factor for 15 hours and processed as described above. ICAM-1 expression was determined by incubation with a 1:1000 dilution of the monoclonal antibody 84H10 (Amac Inc., Westbrook, ME). The cell lines used were passage 4 human umbilical vein endothelial cells (HUVEC), a human epidermal carcinoma cell line (A431), a human melanoma cell line (SK-MEL-2) and a human lung carcinoma cell line (A549). ICAM-1 was induced on all the cell lines, however, tumor necrosis factor was more effective than interleukin-1 in induction of ICAM-1 expression on the cell surface (Figure 4).

Screening antisense oligonucleotides for inhibition of ICAM-1, VCAM-1 or ELAM-1 expression is performed as described above with the exception of pretreatment of cells with the oligonucleotides prior to challenge with the cytokines. An example of antisense oligonucleotide inhibition of ICAM-1 expression is shown in Figure 5. Human umbilical vein endothelial cells (HUVEC) were treated with increasing concentration of oligonucleotide diluted in Opti MEM (GIBCO, Grand Island, NY) containing 8 μM N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) for 4 hours at 37°C to enhance uptake of the oligonucleotides. The medium was removed and replaced with endothelial growth medium (EGM-UV; Clonetics, San Diego, CA) containing the indicated concentration of oligonucleotide for an additional 4 hours. Interleukin-1β was added to the cells at a concentration of 5 units/ml and incubated for 14 hours at 37°C. The cells were quantitated for ICAM-1 expression using a 1:1000 dilution of the monoclonal antibody 84H10 as described above. The oligonucleotides used were:

COMPOUND 1 - (ISIS 1558) a phosphodiester oligonucleotide designed to hybridize with position 64-80 of the mRNA covering the AUG initiation of translation codon having the sequence 5'-TGGGAGCCATAGCGAGGC-3' (SEQ ID NO: 1).

COMPOUND 2 - (ISIS 1570) a phosphorothioate containing oligonucleotide corresponding to the same sequence as COMPOUND 1.

COMPOUND 3 - a phosphorothioate oligonucleotide complementary to COMPOUND 1 and COMPOUND 2 exhibiting the sequence 5'-GCCTCGCTATGGCTCCCA-3' (SEQ ID NO: 81).

COMPOUND 4 - (ISIS 1572) a phosphorothioate containing oligonucleotide designed to hybridize to positions 2190-2210 of the mRNA in the 3' untranslated region containing the sequence 5'-GACACTCAATAAATAGCTGGT-3' (SEQ ID NO: 3).

COMPOUND 5 - (ISIS 1821) a phosphorothioate containing oligonucleotide designed to hybridize to human 5-lipoxygenase mRNA used as a control containing the sequence 5'-CATGGCGCGGGCCGCGGG-3' (SEQ ID NO: 82).

The phosphodiester oligonucleotide targeting the AUG initiation of translation region of the human ICAM-1 mRNA (COMPOUND 1) did not inhibit expression of ICAM-1; however, the corresponding phosphorothioate containing oligonucleotide (COMPOUND 2) inhibited ICAM-1 expression by 70% at a concentration of 0.1 μM and 90% at 1 μM concentration (Figure 4). The increased potency of the phosphorothioate oligonucleotide over the phosphodiester is probably due to increased stability. The sense strand to COMPOUND 2, COMPOUND 3, modestly inhibited ICAM-1 expression at 10 μM. If COMPOUND 2 was prehybridized to COMPOUND 3 prior to addition to the cells, the effects of COMPOUND 2 on ICAM-1 expression were attenuated suggesting that the activity of COMPOUND 2 was due to antisense oligonucleotide effect, requiring hybridization to the mRNA. The antisense oligonucleotide directed against 3' untranslated sequences (COMPOUND 4) inhibited ICAM-1 expression 62% at a concentration of 1 μM (Figure 5). The control oligonucleotide, targeting human 5-lipoxygenase (COMPOUND 5) reduced ICAM-1 expression by 20%. These data demonstrate that oligonucleotides are capable of inhibiting ICAM-1 expression on human umbilical vein endothelial cells and suggest that the inhibition of ICAM-1 expression is due to an antisense activity.

The antisense oligonucleotide COMPOUND 2 at a concentration of 1 μM inhibits expression of ICAM-1 on human umbilical vein endothelial cells stimulated with increasing concentrations of tumor necrosis factor and interleukin-1 (Figure 6). These data demonstrate that the effects of COMPOUND 2 are not specific for interleukin-1 stimulation of cells.

Analogous assays can also be used to demonstrate inhibition of ELAM-1 and VCAM-1 expression by antisense oligonucleotides.

Example 2

A second cellular assay which can be used to demonstrate the effects of antisense oligonucleotides on ICAM-1, VCAM-1 or ELAM-1 expression is a cell adherence assay. Target cells are grown as a monolayer in a multiwell plate, treated with oligonucleotide

followed by cytokine. The adhering cells are then added to the monolayer cells and incubated for 30 to 60 minutes at 37°C and washed to remove nonadhering cells. Cells adhering to the monolayer may be determined either by directly counting the adhering cells or prelabeling the cells with a radioisotope such as ^{51}Cr and quantitating the radioactivity associated with the monolayer as described. Dustin and Springer, *J. Cell Biol.* **1988**, 107, 321-331. Antisense oligonucleotides may target either ICAM-1, VCAM-1 or ELAM-1 in the assay.

An example of the effects of antisense oligonucleotides targeting ICAM-1 mRNA on the adherence of DMSO differentiated HL-60 cells to tumor necrosis factor treated human umbilical vein endothelial cells is shown in Figure 7. Human umbilical vein endothelial cells were grown to 80% confluence in 12 well plates. The cells were treated with 2 μM oligonucleotide diluted in Opti-MEM containing 8 μM DOTMA for 4 hours at 37°C. The medium was removed and replaced with fresh endothelial cell growth medium (EGM-UV) containing 2 μM of the indicated oligonucleotide and incubated 4 hours at 37°C. Tumor necrosis factor, 1 ng/ml, was added to cells as indicated and cells incubated for an additional 19 hours. The cells were washed once with EGM-UV and 1.6 x 10^6 HL-60 cells differentiated for 4 days with 1.3% DMSO added. The cells were allowed to attach for 1 hour at 37°C and gently washed 4 times with Dulbecco's phosphate-buffered saline (D-PBS) warmed to 37°C. Adherent cells were detached from the monolayer by addition of 0.25 ml of cold (4EC) phosphate-buffered saline containing 5 mM EDTA and incubated on ice for 5 minutes. The number of cells removed by treatment with EDTA was determined by counting with a hemocytometer. Endothelial cells detached from the monolayer by EDTA treatment could easily be distinguished from HL-60 cells by morphological differences.

In the absence of tumor necrosis factor, 3% of the HL-60 cells bound to the endothelial cells. Treatment of the endothelial cell monolayer with 1 ng/ml tumor necrosis factor increased the number of adhering cells to 59% of total cells added (Figure 7).

Treatment with the antisense oligonucleotide COMPOUND 2 or the control oligonucleotide COMPOUND 5 did not change the number of cells adhering to the monolayer in the absence of tumor necrosis factor treatment (Figure 7). The antisense oligonucleotide, COMPOUND 2 reduced the number of adhering cells from 59% of total cells added to 17% of the total cells added (Figure 7). In contrast, the control oligonucleotide COMPOUND 5 did not significantly reduce the number of cells adhering to the tumor necrosis factor treated endothelial monolayer, i.e., 53% of total cells added for COMPOUND 5 treated cells versus 59% for control cells.

These data indicate that antisense oligonucleotides are capable of inhibiting ICAM-1 expression on endothelial cells and that inhibition of ICAM-1 expression correlates with a decrease in the adherence of a neutrophil-like cell to the endothelial monolayer in a sequence specific fashion. Because other molecules also mediate adherence of white blood cells to endothelial cells, such as ELAM-1, and VCAM-1 it is not expected that adherence would be completely blocked.

Example 3

Synthesis and characterization of oligonucleotides

Unmodified DNA oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. ß-cyanoethyldiisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-O-methyl phosphorothioate oligonucleotides were synthesized using 2'-O-methyl ß-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham MA) and the standard cycle

for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. The 3'-base used to start the synthesis was a 2'-deoxyribonucleotide.

2'-fluoro phosphorothioate oligonucleotides were synthesized using 5'-dimethoxytrityl-3'-phosphoramidites and prepared as disclosed in U.S. patent application Serial No. 463,358, filed January 11, 1990, and 566,977, filed August 13, 1990, which are assigned to the same assignee as the instant application and which are incorporated by reference herein. The 2'-fluoro oligonucleotides were prepared using phosphoramidite chemistry and a slight modification of the standard DNA synthesis protocol: deprotection was effected using methanolic ammonia at room temperature.

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM Tris-borate buffer, pH 7.0. Oligodeoxynucleotides and phosphorothioate oligonucleotides were judged from electrophoresis to be greater than 80% full length material.

RNA oligonucleotide synthesis was performed on an ABI model 380B DNA synthesizer. The standard synthesis cycle was modified by increasing the wait step after the pulse delivery of tetrazole to 900 seconds. The bases were deprotected by incubation in methanolic ammonia overnight. Following base deprotections the oligonucleotides were dried *in vacuo*. The t-butyldimethylsilyl protecting the 2' hydroxyl was removed by incubating the oligonucleotide in 1 M tetrabutylammonium-fluoride in tetrahydrofuran overnight. The RNA oligonucleotides were further purified on C_{18} Sep-Pak cartridges (Waters, Division of Millipore Corp., Milford MA) and ethanol precipitated.

The relative amounts of phosphorothioate and phosphodiester linkages obtained by this synthesis were periodically checked by ^{31}P NMR spectroscopy. The spectra were obtained at ambient

temperature using deuterium oxide or dimethyl sulfoxide-d_6 as solvent. Phosphorothioate samples typically contained less than one percent of phosphodiester linkages.

Secondary evaluation was performed with oligonucleotides purified by trityl-on HPLC on a PRP-1 column (Hamilton Co., Reno, Nevada) using a gradient of acetonitrile in 50 mM triethylammonium acetate, pH 7.0 (4% to 32% in 30 minutes, flow rate = 1.5 ml/min). Appropriate fractions were pooled, evaporated and treated with 5% acetic acid at ambient temperature for 15 minutes. The solution was extracted with an equal volume of ethyl acetate, neutralized with ammonium hydroxide, frozen and lyophilized. HPLC-purified oligonucleotides were not significantly different in potency from precipitated oligonucleotides, as judged by the ELISA assay for ICAM-1 expression.

Example 4

Cell culture and treatment with oligonucleotides

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (Bethesda MD). Cells were grown in Dulbecco's Modified Eagle's Medium (Irvine Scientific, Irvine CA) containing 1 gm glucose/liter and 10% fetal calf serum (Irvine Scientific). Human umbilical vein endothelial cells (HUVEC) (Clonetics, San Diego CA) were cultured in EGM-UV medium (Clonetics). HUVEC were used between the second and sixth passages. Human epidermal carcinoma A431 cells were obtained from the American Type Culture Collection and cultured in DMEM with 4.5 g/l glucose. Primary human keratinocytes were obtained from Clonetics and grown in KGM (Keratinocyte growth medium, Clonetics).

Cells grown in 96-well plates were washed three times with Opti-MEM (GIBCO, Grand Island, NY) prewarmed to 37°C. 100 µl of Opti-MEM containing either 10 µg/ml N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA, Bethesda Research Labs, Bethesda MD) in the case of HUVEC cells or 20 µg/ml DOTMA in the case of A549 cells was added to each well. Oligonucleotides were

sterilized by centrifugation through 0.2 μm Centrex cellulose acetate filters (Schleicher and Schuell, Keene, NH). Oligonucleotides were added as 20x stock solution to the wells and incubated for 4 hours at 37°C. Medium was removed and replaced with 150 μl of the appropriate growth medium containing the indicated concentration of oligonucleotide. Cells were incubated for an additional 3 to 4 hours at 37°C then stimulated with the appropriate cytokine for 14 to 16 hours, as indicated. ICAM-1 expression was determined as described in Example 1. The presence of DOTMA during the first 4 hours incubation with oligonucleotide increased the potency of the oligonucleotides at least 100-fold. This increase in potency correlated with an increase in cell uptake of the oligonucleotide.

Example 5

ELISA screening of additional antisense oligonucleotides for activity against ICAM-1 gene expression in Interleukin-1ß-stimulated cells

Antisense oligonucleotides were originally designed that would hybridize to five target sites on the human ICAM-1 mRNA. Oligonucleotides were synthesized in both phosphodiester (P=O; ISIS 1558, 1559, 1563, 1564 and 1565)

and phosphorothioate (P=S; ISIS 1570, 1571, 1572, 1573, and 1574) forms. The oligonucleotides are shown in Table 1.

Table 1

ANTISENSE OLIGONUCLEOTIDES WHICH TARGET HUMAN ICAM-1

ISIS NO.	SEQ ID NO.	TARGET REGION	MODIFICATION
1558	1	AUG Codon (64-81)	P=O
1559	2	5'-Untranslated (32-49)	P=O
1563	3	3'-Untranslated (2190-3010)	P=O
1564	4	3'-Untranslated (2849-2866)	P=O
1565	5	Coding Region (1378-1395)	P=O
1570	1	AUG Codon (64-81)	P=S
1571	2	5'-Untranslated (32-49)	P=S
1572	3	3'-Untranslated (2190-3010)	P=S
1573	4	3'-Untranslated (2849-2866)	P=S
1574	5	Coding Region (1378-1395)	P=S
1930	6	5'-Untranslated (1-20)	P=S
1931	7	AUG Codon (55-74)	P=S
1932	8	AUG Codon (72-91)	P=S
1933	9	Coding Region (111-130)	P=S
1934	10	Coding Region (351-370)	P=S
1935	11	Coding Region (889-908)	P=S
1936	12	Coding Region (1459-1468)	P=S
1937	13	Termination Codon (1651-1687)	P=S
1938	14	Termination Codon (1668-1687)	P=S
1939	15	3'-Untranslated (1952-1971)	P=S
1940	16	3'-Untranslated (2975-2994)	P=S
2149	17	AUG Codon (64-77)	P=S
2163	18	AUG Codon (64-75)	P=S
2164	19	AUG Codon (64-73)	P=S
2165	20	AUG Codon (66-80)	P=S
2173	21	AUG Codon (64-79)	P=S
2302	22	3'-Untranslated (2114-2133)	P=S

ISIS NO.	SEQ ID NO.	TARGET REGION	MODIFICATION
2303	23	3'-Untranslated (2039-2058)	P=S
2304	24	3'-Untranslated (1895-1914)	P=S
2305	25	3'-Untranslated (1935-1954)	P=S
2307	26	3'-Untranslated (1976-1995)	P=S
2634	1	AUG-Codon (64-81)	2'-fluoro A,C & U; P=S
2637	15	3'-Untrans(1952-1971)	2'-fluoro A, C & U;
2691	1	AUG Codon (64-81)	P=O, except last 3 bases, P=S
2710	15	3'-Untrans. (1952-1971)	2'-O-methyl; P=O
2711	1	AUG Codon (64-81)	2'-O-methyl; P=O
2973	15	3'-Untrans.(1952-1971)	2'-O-methyl; P=S
2974	1	AUG Codon (64-81)	2'-O-methyl; P=S
3064	27	5'-CAP (32-51)	P=S; G & C added as spacer to 3'
3067	84	5'-CAP (32-51)	P=S
3222	84	5'-CAP (32-51)	2'-O-methyl; P=O
3224	84	5'-CAP (32-51)	2'-O-methyl; P=S
3581	85	3'-Untranslated (1959-1978)	P=S

Inhibition of ICAM-1 expression on the surface of interleukin-1ß-stimulated cells by the oligonucleotides was determined by ELISA assay as described in Example 1. The oligonucleotides were tested in two different cell lines. None of the phosphodiester oligonucleotides inhibited ICAM-1 expression. This is probably due to the rapid degradation of phosphodiester oligonucleotides in cells. Of the five phosphorothioate oligonucleotides, the most active was ISIS 1570, which hybridizes to the AUG translation initiation codon. A 2'-o-methyl phosphorothioate oligonucleotide, ISIS 2974, was approximately threefold less effective than ISIS 1570 in inhibiting ICAM-1 expression in HUVEC and A549 cells. A 2'-fluoro oligonucleotide, ISIS 2634, was also less effective.

Based on the initial data obtained with the five original targets, additional oligonucleotides were designed which would hybridize with the ICAM-1 mRNA. The antisense oligonucleotide (ISIS 3067) which hybridizes to the predicted transcription initiation site (5' cap site) was approximately as active in IL-1ß-stimulated cells as the oligonucleotide that hybridizes to the AUG codon (ISIS 1570), as shown in Figure 8. ISIS 1931 and 1932 hybridize 5' and 3', respectively, to the AUG translation initiation codon. All three oligonucleotides that hybridize to the AUG region inhibit ICAM-1 expression, though ISIS 1932 was slightly less active than ISIS 1570 and ISIS 1931. Oligonucleotides which hybridize to the coding region of ICAM-1 mRNA (ISIS 1933, 1934, 1935, 1574 and 1936) exhibited weak activity. Oligonucleotides that hybridize to the translation termination codon (ISIS 1937 and 1938) exhibited moderate activity.

Surprisingly, the most active antisense oligonucleotide was ISIS 1939, a phosphorothioate oligonucleotide targeted to a sequence in the 3'- untranslated region of ICAM-1 mRNA (see Table 1). Other oligonucleotides having the same sequence were tested, 2'-O-methyl (ISIS 2973) and 2'-fluoro (ISIS 2637); however, they did not exhibit this level of activity. Oligonucleotides targeted to other 3' untranslated sequences (ISIS 1572, 1573 and 1940) were also not as active as ISIS-1939. In fact, ISIS 1940, targeted to the polyadenylation signal, did not inhibit ICAM-1 expression.

Because ISIS 1939 proved unexpectedly to exhibit the greatest antisense activity of the original 16 oligonucleotides tested, other oligonucleotides were designed to hybridize to sequences in the 3'-untranslated region of ICAM-1 mRNA (ISIS 2302, 2303, 2304, 2305, and 2307, as shown in Table 1). ISIS 2307, which hybridizes to a site only five bases 3' to the ISIS 1939 target, was the least active of the series (Figure 8). ISIS 2302, which hybridizes to the ICAM-1 mRNA at a position 143 bases 3' to the ISIS 1939 target, was the most active of the series, with activity comparable to that of ISIS 1939. Examination of the

predicted RNA secondary structure of the human ICAM-1 mRNA 3'-untranslated region (according to M. Zuker, *Science* **1989**, 244, 48-52) revealed that both ISIS 1939 and ISIS 2302 hybridize to sequences predicted to be in a stable stem-loop structure. Current dogma suggests that regions of RNA secondary structure should be avoided when designing antisense oligonucleotides. Thus, ISIS 1939 and ISIS 2302 would not have been predicted to inhibit ICAM-1 expression.

The control oligonucleotide ISIS 1821 did inhibit ICAM-1 expression in HUVEC cells with activity comparable to that of ISIS 1934; however, in A549 cells ISIS 1821 was less effective than ISIS 1934. The negative control, ISIS 1821, was found to have a small amount of activity against ICAM expression, probably due in part to its ability to hybridize (12 of 13 base match) to the ICAM-1 mRNA at a position 15 bases 3' to the AUG translation initiation codon.

These studies indicate that the AUG translation initiation codon and specific 3'-untranslated sequences in the ICAM-1 mRNA were the most susceptible to antisense oligonucleotide inhibition of ICAM-1 expression.

In addition to inhibiting ICAM-1 expression in human umbilical vein cells and the human lung carcinoma cells (A549), ISIS 1570, ISIS 1939 and ISIS 2302 were shown to inhibit ICAM-1 expression in the human epidermal carcinoma A431 cells and in primary human keratinocytes (shown in Figure 9). These data demonstrate that antisense oligonucleotides are capable of inhibiting ICAM-1 expression in several human cell lines. Furthermore, the rank order potency of the oligonucleotides is the same in the four cell lines examined. The fact that ICAM-1 expression could be inhibited in primary human keratinocytes is important because epidermal keratinocytes are an intended target of the antisense nucleotides.

Example 6

Antisense oligonucleotide inhibition of ICAM-1 expression in cells

stimulated with other cytokines

Two oligonucleotides, ISIS 1570 and ISIS 1939, were tested for their ability to inhibit TNF-α and IFN-α-induced ICAM-1 expression. Treatment of A549 cells with 1 μM antisense oligonucleotide inhibited IL-1ß, TNF-α and IFN-α-induced ICAM-1 expression in a sequence-specific manner. The antisense oligonucleotides inhibited IL-1ß and TNF-α-induced ICAM-1 expression to a similar extent, while IFN-α-induced ICAM-1 expression was more sensitive to antisense inhibition. The control oligonucleotide, ISIS 1821, did not significantly inhibit IL-1ß- or TNF-α-induced ICAM-1 expression and inhibited IFN-α-induced ICAM-1 expression slightly, as follows:

Antisense Oligonucleotide
(% Control Expression)

Cytokine	ISIS 1570	ISIS 1939	ISIS 1821
3 U/ml IL-1â	56.6 " 2.9	38.1 " 3.2	95 " 6.6
1 ng/ml TNF-á	58.1 " 0.9	37.6 " 4.1	103.5 " 8.2
100 U/ml gamma-IFN	38.9 " 3.0	18.3 " 7.0	83.0 " 3.5

Example 7

Antisense effects are abolished by sense strand controls

The antisense oligonucleotide inhibition of ICAM-1 expression by the oligonucleotides ISIS 1570 and ISIS 1939 could be reversed by hybridization of the oligonucleotides with their respective sense strands. The phosphorothioate sense strand for ISIS 1570 (ISIS 1575), when applied alone, slightly enhanced IL-1ß-induced ICAM-1 expression. Premixing ISIS 1570 with ISIS 1575 at equal molar concentrations, prior to addition to the cells, blocked the effects of ISIS 1570. The complement to ISIS 1939 (ISIS 2115) enhanced ICAM-1 expression by 46% when added to the cells alone. Prehybridization of ISIS 2115 to ISIS 1939 completely blocked the inhibition of ICAM-1 expression by ISIS

1939.

Example 8

Measurement of oligonucleotide Tm (dissociation temperature of oligonucleotide from target)

To determine if the potency of the inhibition of ICAM-1 expression by antisense oligonucleotides was due to their affinity for their target sites, thermodynamic measurements were made for each of the oligonucleotides. The antisense oligonucleotides (synthesized as phosphorothioates) were hybridized to their complementary DNA sequences (synthesized as phosphodiesters). Absorbance vs. temperature profiles were measured at 4 μM each strand oligonucleotide in 100 mM Na+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0. Tm's and free energies of duplex formation were obtained from fits of data to a two-state model with linear sloping baselines (Petersheim, M. and D.H. Turner, *Biochemistry* **1983**, 22, 256-263). Results are averages of at least three experiments.

When the antisense oligonucleotides were hybridized to their complementary DNA sequences (synthesized as phosphodiesters), all of the antisense oligonucleotides with the exception of ISIS 1940 exhibited a Tm of at least 50°C. All the oligonucleotides should therefore be capable of hybridizing to the target ICAM-1 mRNA if the target sequences were exposed. Surprisingly, the potency of the antisense oligonucleotide did not correlate directly with either Tm or $\AA GE_{37}$. The oligonucleotide with the greatest biological activity, ISIS 1939, exhibited a Tm which was lower than that of the majority of the other oligonucleotides. Thus, hybridization affinity is not sufficient to ensure biological activity.

Example 9

Effect of oligonucleotide length on antisense inhibition of ICAM-1 expression

The effect of oligonucleotide length on antisense activity

was tested using truncated versions of ISIS 1570 (ISIS 2165, 2173, 2149, 2163 and 2164) and ISIS 1939 (ISIS 2540, 2544, 2545, 2546, 2547 and 2548). In general, antisense activity decreased as the length of the oligonucleotides decreased. Oligonucleotides 16 bases in length exhibited activity slightly less than 18 base oligonucleotides. Oligonucleotides 14 bases in length exhibited significantly less activity, and oligonucleotides 12 or 10 bases in length exhibited only weak activity. Examination of the relationship between oligonucleotide length and Tm and antisense activity reveals that a sharp transition occurs between 14 and 16 bases in length, while Tm increases linearly with length (Figure 10).

Example 10

Specificity of antisense inhibition of ICAM-1

The specificity of the antisense oligonucleotides ISIS 1570 and ISIS 1939 for ICAM-1 was evaluated by immunoprecipitation of ^{35}S-labelled proteins. A549 cells were grown to confluence in 25 cm^2 tissue culture flasks and treated with antisense oligonucleotides as described in Example 4. The cells were stimulated with interleukin-1ß for 14 hours, washed with methionine-free DMEM plus 10% dialyzed fetal calf serum, and incubated for 1 hour in methionine-free medium containing 10% dialyzed fetal calf serum, 1 µM oligonucleotide and interleukin-1ß as indicated. ^{35}S-Methionine/cysteine mixture (Tran^{35}S-label, purchased from ICN, Costa Mesa, CA) was added to the cells to an activity of 100 µCi/ml and the cells were incubated an additional 2 hours. Cellular proteins were extracted by incubation with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate and 2 mM EDTA (0.5 ml per well) at 4°C for 30 minutes. The extracts were clarified by centrifugation at 18,000 x g for 20 minutes. The supernatants were preadsorbed with 200 µl protein G-Sepharose beads (Bethesda Research Labs, Bethesda MD) for 2 hours at 4°C, divided equally and incubated with either 5 µg ICAM-1 monoclonal antibody (purchased from AMAC Inc., Westbrook ME) or HLA-A,B

antibody (W6/32, produced by murine hybridoma cells obtained from the American Type Culture Collection, Bethesda, MD) for 15 hours at 4°C. Immune complexes were trapped by incubation with 200 µl of a 50% suspension of protein G-Sepharose (v/v) for 2 hours at 4°C, washed 5 times with lysis buffer and resolved on an SDS-polyacrylamide gel. Proteins were detected by autoradiography.

Treatment of A549 cells with 5 units/ml of interleukin-1ß was shown to result in the synthesis of a 95-100 kDa protein migrating as a doublet which was immunoprecipitated with the monoclonal antibody to ICAM-1. The appearance as a doublet is believed to be due to differently glycosylated forms of ICAM-1. Pretreatment of the cells with the antisense oligonucleotide ISIS 1570 at a concentration of 1 µM decreased the synthesis of ICAM-1 by approximately 50%, while 1 µM ISIS 1939 decreased ICAM-1 synthesis to near background. Antisense oligonucleotide ISIS 1940, inactive in the ICAM-1 ELISA assay (Examples 1 and 5) did not significantly reduce ICAM-1 synthesis. None of the antisense oligonucleotides hybridizable with ICAM-1 targets had a demonstrable effect on HLA-A, B synthesis, demonstrating the specificity of the oligonucleotides for ICAM-1. Furthermore, the proteins which nonspecifically precipitated with the ICAM-1 antibody and protein G-Sepharose were not significantly affected by treatment with the antisense oligonucleotides.

Example 11

Screening of additional antisense oligonucleotides for activity against ICAM-1 by cell adhesion assay

Human umbilical vein endothelial (HUVEC) cells were grown and treated with oligonucleotides as in Example 4. Cells were treated with either ISIS 1939, ISIS 1940, or the control oligonucleotide ISIS 1821 for 4 hours, then stimulated with TNF-α for 20 hours. Basal HUVEC minimally bound HL-60 cells, while TNF-stimulated HUVEC bound 19% of the total cells added. Pretreatment of the HUVEC monolayer with 0.3 µM ISIS 1939 reduced the adherence of HL-60 cells to basal levels, as shown in Figure 11. The

control oligonucleotide, ISIS 1821, and ISIS 1940 reduced the percentage of cells adhering from 19% to 9%. These data indicate that antisense oligonucleotides targeting ICAM-1 may specifically decrease adherence of a leukocyte-like cell line (HL-60) to TNF-α-treated HUVEC.

Example 12

ELISA screening of antisense oligonucleotides for activity against ELAM-1 gene expression

Primary human umbilical vein endothelial (HUVEC) cells, passage 2 to 5, were plated in 96-well plates and allowed to reach confluence. Cells were washed three times with Opti-MEM (GIBCO, Grand Island NY). Cells were treated with increasing concentrations of oligonucleotide diluted in Opti-MEM containing 10 µg/ml DOTMA solution (Bethesda Research Labs, Bethesda, MD) for 4 hours at 37°C. The medium was removed and replaced with EGM-UV (Clonetics, San Diego CA) plus oligonucleotide. Tumor necrosis factor α was added to the medium (2.5 ng/ml) and the cells were incubated an additional 4 hours at 37°C.

ELAM-1 expression was determined by ELISA. Cells were gently washed three times with Dulbecco's phosphate-buffered saline (D-PBS) prewarmed to 37°C. Cells were fixed with 95% ethanol at 4°C for 20 minutes, washed three times with D-PBS and blocked with 2% BSA in D-PBS. Cells were incubated with ELAM-1 monoclonal antibody BBA-1 (R&D Systems, Minneapolis MN) diluted to 0.5 µg/ml in D-PBS containing 2% BSA for 1 hour at 37°C. Cells were washed three times with D-PBS and the bound ELAM-1 antibody detected with biotinylated goat anti-mouse secondary antibody followed by ß-galactosidase-conjugated streptavidin as described in Example 1.

The activity of antisense phosphorothioate oligonucleotides which target 11 different regions on the ELAM-1 cDNA and two oligonucleotides which target ICAM-1 (as controls) was determined using the ELAM-1 ELISA. The oligonucleotide and targets are shown in Table 2.

Table 2

ANTISENSE OLIGONUCLEOTIDES WHICH TARGET HUMAN ELAM-1

ISIS NO.	SEQ ID NO.	TARGET REGION	MODIFICATION
1926	28	AUG Codon (143-164)	P=S
2670	29	3'-Untranslated (3718-3737)	P=S
2673	30	3'-Untranslated (2657-2677)	P=S
2674	31	3'-Untranslated (2617-2637)	P=S
2678	32	3'-Untranslated (3558-3577)	P=S
2679	33	5'-Untranslated (41-60)	P=S
2680	34	3'-Untranslated (3715-3729)	P=S
2683	35	AUG Codon (143-163)	P=S
2686	36	AUG Codon (149-169)	P=S
2687	37	5'-Untranslated (18-37)	P=S
2693	38	3'-Untranslated (2760-2788)	P=S
2694	39	3'-Untranslated (2934-2954)	P=S

In contrast to what was observed with antisense oligonucleotides targeted to ICAM-1 (Example 5), the most potent oligonucleotide modulator of ELAM-1 activity (ISIS 2679) was hybridizable with specific sequences in the 5'-untranslated region of ELAM-1. ISIS 2687, an oligonucleotide which hybridized to sequences ending three bases upstream of the ISIS 2679 target, did not show significant activity (Figure 12). Therefore, ISIS 2679 hybridizes to a unique site on the ELAM-1 mRNA, which is uniquely sensitive to inhibition with antisense oligonucleotides. The sensitivity of this site to inhibition with antisense oligonucleotides was not predictable based upon RNA secondary structure predictions or information in the literature.

Example 13

ELISA screening of additional antisense oligonucleotides for activity against ELAM-1 gene expression

Inhibition of ELAM-1 expression by eighteen antisense phosphorothioate oligonucleotides was determined using the ELISA assay as described in Example 12. The target sites of these oligonucleotides on the ELAM-1 mRNA are shown in Figure 13. The sequence and activity of each oligonucleotide against ELAM-1 are shown in Table 3. The oligonucleotides indicated by an asterisk (*) have IC50's of approximately 50 nM or below and are preferred. IC50 indicates the dosage of oligonucleotide, which results in 50% inhibition of ELAM-1 expression.

TABLE 3

INHIBITION OF HUMAN ELAM-1 EXPRESSION BY ANTISENSE OLIGONUCLEOTIDES

ELAM-1 expression is given as % of control

ISIS#	SEQ ID NO:	POSITION	SEQUENCE	VCAM-1 EXPRESSION	
				30 nM oligo	50 nM oligo
*4764	52	5'-UTR 1-19	GAAGTCAGCCAAGAACAGCT	70.2	50.2
2687	37	5'-UTR 17-36	TATAGGAGTTTTGATGTGAA	91.1	73.8
*2679	33	5'-UTR 40-59	CTGCTGCCTCTGTCTCAGGT	6.4	6.0
*4759	53	5'-UTR 64-83	ACAGGATCTCTCAGGTGGGT	30.0	20.2
*2683	35	AUG 143-163	AATCATGACTTCAAGAGTTCT	47.9	48.5
*2686	36	AUG 148-168	TGAAGCAATCATGACTTCAAG	51.1	46.9
*4756	54	I/E 177-196	CCAAAGTGAGAGCTGAGAGA	53.9	35.7
4732	55	Coding 1936-1955	CTGATTCAAGGCTTTGGCAG	68.5	55.3
*4730	56	I/E 3'UTR 2006-2025	TCCCCAGATGCACCTGTTT	14.1	2.3
*4729	57	3'-UTR 2063-2082	GGGCCAGAGACCCGAGGAGA	49.4	46.3
*2674	31	3'-UTR 2617-2637	CACAATCCTTAAGAACTCTTT	33.5	28.1
2673	30	3'-UTR 2656-2676	GTATGGAAGATTATAATATAT	58.9	53.8
2694	39	3'-UTR 2933-2953	GACAATATACAAACCTTCCAT	72.0	64.6
*4719	58	3'-UTR 2993-3012	ACGTTTGGCCTCATGGAAGT	36.8	34.7

ISIS#	SEQ ID NO:	POSITION	SEQUENCE	VCAM-1 EXPRESSION	
				30 nM oligo	50 nM oligo
4720	59	3'-UTR 3093-3112	GGAATGCAAAGCACATCCAT	63.5	70.6
*2678	32	3'-UTR 3557-3576	ACCTCTGCTGTTCTGATCCT	24.9	15.3
2670	29	3'-UTR 3717-3736	ACCACACTGGTATTTCACAC	72.2	67.2

I/E indicates Intron/Exon junction

Oligonucleotides with IC50's of approximately 50 nM or below are indicated by an asterisk (*).

An additional oligonucleotide targeted to the 3'-untranslated region (ISIS 4728) did not inhibit ELAM expression.

Example 14

ELISA screening of antisense oligonucleotides for activity against VCAM-1 gene expression

Inhibition of VCAM-1 expression by fifteen antisense phosphorothioate oligonucleotides was determined using the ELISA assay approximately as described in Example 12, except that cells were stimulated with TNF-á for 16 hours and VCAM-1 expression was detected by a VCAM-1 specific monoclonal antibody (R & D Systems, Minneapolis, MN) used at 0.5 µg/ml. The target sites of these oligonucleotides on the VCAM-1 mRNA are shown in Figure 14. The sequence and activity of each oligonucleotide against VCAM-1 are shown in Table 4. The oligonucleotides indicated by an asterisk (*) have IC50's of approximately 50 nM or below and are preferred. IC50 indicates the dosage of oligonucleotide which results in 50% inhibition of VCAM-1 expression.

Table 4

INHIBITION OF HUMAN VCAM-1 EXPRESSION BY ANTISENSE OLIGONUCLEOTIDES

VCAM-1 expression is given as % of control

ISIS#	SEQ ID NO:	POSITION		SEQUENCE	VCAM-1 EXPRESSION	
					30 nM oligo	50 nM oligo
*5884	60	5'-UTR	1-19	CGATGCAGATACCGCGGAGT	79.2	37.2
3791	61	5'-UTR	38-58	CCTGGGAGGGTATTCAGCT	92.6	58.0
5862	62	5'-UTR	48-68	CCTGTGTGTGCCTGGGAGGG	115.0	3.5
*3792	63	AUG	110-129	GGCATTTTAAGTTGCTGTCG	68.7	33.7
5863	64	CODING	745-764	CAGCCTGCCTTACTGTGGGC	95.8	66.7
*5874	65	CODING	1032-1052	CTTGAACAATTAATTCCACCT	66.5	35.3
5885	66	E/I 1633-1649+intron		TTACCATTGACATAAAGTGTT	84.4	52.4
*5876	67	CODING	2038-2057	CTGTGTCTCCTGTCTCCGCT	43.5	26.6
*5875	68	CODING	2183-2203	GTCTTTGTTGTTTTCTCTTCC	59.2	34.8
3794	69	TERMIN.	2344-2362	TGAACATATCAAGCATTAGC	75.3	52.6
*3800	70	3'-UTR	2620-2639	GCAATCTTGCTATGGCATAA	64.4	47.7
*3805	71	3'-UTR	2826-2845	CCCGGCATCTTTACAAAACC	7.7	44.9
*3801	50	3'-UTR	2872-2892	AACCCAGTGCTCCCTTTGCT	36.5	21.3
*5847	72	3'-UTR	2957-2976	AACATCTCCGTACCATGCCA	51.8	24.6
*3804	51	3'-UTR	3005-3024	GGCCACATTGGGAAAGTTGC	55.1	29.3

E/I indicates exon/intron junction

Oligonucleotides with IC50's of approximately 50 nM or below are indicated by an asterisk (*).

Example 15

ICAM-1 expression in C8161 human melanoma cells

Human melanoma cell line C8161 (a gift of Dr. Dan Welch, Hershey Medical Center) was derived from an abdominal wall metastasis from a patient with recurrent malignant melanoma. These cells form multiple metastases in lung, subcutis, spleen, liver and regional lymph nodes after subcutaneous, intradermal and intravenous injection into athymic nude mice. Cells were grown in DMA-F12 medium containing 10% fetal calf serum and were passaged using 2 mM EDTA.

Exposure of C8161 cells to TNF-α resulted in a six-fold increase in cell surface expression of ICAM-1 and an increase in ICAM-1 mRNA levels in these cells. ICAM-1 expression on the cell surface was measured by ELISA. Cells were treated with increasing concentrations of antisense oligonucleotides in the presence of 15 μg/ml Lipofectin for 4 hours at 37°C. ICAM-1 expression was induced by incubation with 5 ng/ml TNF-α for 16 hours. Cells were washed 3x in DPBS and fixed for 20 minutes in 2% formaldehyde. Cells were washed in DPBS, blocked with 2% BSA for 1 hour at 37°C and incubated with ICAM-1 monoclonal antibody 84H10 (AMAC, Inc., Westbrook, ME). Detection of bound antibody was determined by incubation with a biotinylated goat anti-mouse IgG followed by incubation with ß-galactosidase-conjugated streptavidin and developed with chlorophenol red-ß-D-galactopyranoside and quantified by absorbance at 575 nm. ICAM-1 mRNA levels were measured by Northern blot analysis.

Example 16

Oligonucleotide inhibition of ICAM-1 expression in C8161 human melanoma cells

As shown in Figure 15, antisense oligonucleotides ICAM 1570

(SEQ ID NO: 1), ISIS 1939 (SEQ ID NO: 15) and ISIS 2302 (SEQ ID NO: 22) targeted to ICAM-1 decreased cell surface expression of ICAM-1 (detected by ELISA as in Example 16). ISIS 1822, a negative control oligonucleotide complementary to 5-lipoxygenase, did not affect ICAM-1 expression. The data were expressed as percentage of control activity, calculated as follows: (ICAM-1 expression for oligonucleotide-treated, cytokine-induced cells)-(basal ICAM-1 expression)/(ICAM-1 cytokine-induced expression)-(basal ICAM-1 expression) x 100.

ISIS 1939 (SEQ ID NO: 15) and ISIS 2302 (SEQ ID NO: 22) markedly reduced ICAM-1 mRNA levels (detected by Northern blot analysis), but ISIS 1570 (SEQ ID NO: 1) decreased ICAM-1 mRNA levels only slightly.

Example 17

Experimental metastasis assay

To evaluate the role of ICAM-1 in metastasis, experimental metastasis assays were performed by injecting 1 x 10^5 C8161 cells into the lateral tail vein of athymic nude mice. Treatment of C8161 cells with the cytokine TNF-α and interferon α has previously been shown to result in an increased number of lung metastases when cells were injected into nude mice [Miller, D.E. and Welch, D.R., *Proc. Am. Assoc. Cancer Res.* **1990**, 13, 353].

After 4 weeks, mice were sacrificed, organs were fixed in Bouin's fixative and metastatic lesions on lungs were scored with the aid of a dissecting microscope. Four-week-old female athymic nude mice (Harlan Sprague Dawley) were used. Animals were maintained under the guidelines of the NIH. Groups of 4-8 mice each were tested in experimental metastasis assays.

Example 18

Antisense oligonucleotides ISIS 1570 and ISIS 2302 decrease metastatic potential of C8161 cells

Treatment of C8161 cells with antisense oligonucleotides ISIS 1570 and ISIS 2302, complementary to ICAM-1, was performed in

the presence of the cationic lipid, Lipofectin (Gibco/BRL, Gaithersburg, MD). Antisense oligonucleotides were synthesized as described in Example 3. Cells were seeded in 60 mm tissue culture dishes at 10^6 cells/ml and incubated at 37°C for 3 days, washed with OPTI-MEM (Gibco/BRL) 3 times and 100 µl of OPTI-MEM medium was added to each well. 0.5 µM oligonucleotide and 15 µg/ml lipofectin were mixed at room temperature for 15 minutes. 25 µl of the oligonucleotide-lipofectin mixture was added to the appropriate dishes and incubated at 37°C for 4 hours. The oligonucleotide-lipofectin mixture was removed and replaced with DME-F12 medium containing 10% fetal calf serum. After 4 hours, 500 U/ml TNF-α was added to the appropriate wells and incubated for 18 hours at which time cells were removed from the plates, counted and injected into athymic nude mice.

Treatment of C8161 cells with ISIS 1570 (SEQ ID NO: 1) or ISIS 2302 (SEQ ID NO: 22) decreased the metastatic potential of these cells, and eliminated the enhanced metastatic ability of C8161 which resulted from TNF-á treatment. Data are shown in Table 5.

Table 5

EFFECT OF ANTISENSE OLIGONUCLEOTIDES TO ICAM-1 ON EXPERIMENTAL METASTASIS OF HUMAN MELANOMA CELL LINE C8161

Treatment	No. Lung Metastases per Mouse (Mean ± S.E.M.)
Lipofectin only	64 ± 13
Lipofectin + TNF-á	81 ± 8
ISIS-1570 + Lipofectin	38 ± 15
ISIS-2302 + Lipofectin	23 ± 6
ISIS-1570 + Lipofectin + TNF-á	49 ± 6
ISIS-2302 + Lipofectin + TNF-á	31 ± 8

Example 19

Murine models for testing antisense oligonucleotides against ICAM-1

Many conditions which are believed to be mediated by intercellular adhesion molecules are not amenable to study in humans. For example, allograft rejection is a condition which is likely to be ameliorated by interference with ICAM-1 expression, but clearly this must be evaluated in animals rather than human transplant patients. Another such example is inflammatory bowel disease, and yet another is neutrophil migration (infiltration). These conditions can be tested in animal models, however, such as the mouse models used here.

Oligonucleotide sequences for inhibiting ICAM-1 expression in murine cells were identified. Murine ICAM-1 has approximately 50% homology with the human ICAM-1 sequence; a series of oligonucleotides which target the mouse ICAM-1 mRNA sequence were designed and synthesized, using information gained from evaluation of oligonucleotides

targeted to human ICAM-1. These oligonucleotides were screened for activity using an immunoprecipitation assay.

Murine DCEK-ICAM-1 cells (a gift from Dr. Adrienne Brian, University of California at San Diego) were treated with 1 μM of oligonucleotide in the presence of 20 μg/ml DOTMA/DOPE solution for 4 hours at 37°C. The medium was replaced with methionine-free medium plus 10% dialyzed fetal calf serum and 1 μM antisense oligonucleotide. The cells were incubated for 1 hour in methionine-free medium, then 100 μCi/ml ^{35}S-labeled methionine/cysteine mixture was added to the cells. Cells were incubated an additional 2 hours, washed 4 times with PBS, and extracted with buffer containing 20 mM Tris, pH 7.2, 20 mM KCl, 5 mM EDTA, 1% Triton X-100, 0.1 mM leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. ICAM-1 was immunoprecipitated from the extracts by incubating with a murine-specific ICAM-1 antibody (YN1/1.7.4) followed by protein G-sepharose. The immunoprecipitates were analyzed by SDS-PAGE and autoradiographed. Phosphorothioate oligonucleotides ISIS 3066 and 3069, which target the AUG codon of mouse ICAM-1, inhibited ICAM-1 synthesis by 48% and 63%, respectively, while oligonucleotides ISIS 3065 and ISIS 3082, which target sequences in the 3'-untranslated region of murine ICAM-1 mRNA inhibited ICAM-1 synthesis by 47% and 97%, respectively. The most active antisense oligonucleotide against mouse ICAM-1 was targeted to the 3'-untranslated region. ISIS 3082 was evaluated further based on these results; this 20-mer phosphorothioate oligonucleotide comprises the sequence (5' to 3') TGC ATC CCC CAG GCC ACC AT (SEQ ID NO: 83).

Example 20

Antisense oligonucleotides to ICAM-1 reduce inflammatory bowel disease in murine model system

A mouse model for inflammatory bowel disease (IBD) has recently been developed. Okayasu et al., *Gastroenterology* **1990**, 98, 694-702. Administration of dextran sulfate to mice induces colitis that mimics human IBD in almost every detail. Dextran sulfate-induced IBD and human IBD have subsequently been closely compared at the histological level and the mouse model has been found to be an extremely reproducible and reliable model. It is used here to test the effect of ISIS 3082, a 20-base phosphorothioate antisense oligonucleotide which is complementary to the 3' untranslated region of the murine ICAM-1.

Female Swiss Webster mice (8 weeks of age) weighing approximately 25 to 30 grams are kept under standard conditions. Mice are allowed to acclimate for at least 5 days before initiation of experimental procedures. Mice are given 5% dextran sulfate sodium in their drinking water (available ad libitum) for 5 days. Concomitantly, ISIS 3082 oligonucleotide in pharmaceutical carrier, carrier alone (negative control) or TGF-ß (known to protect against dextran sulfate-mediated colitis in mice) is administered. ISIS 3082 was given as daily subcutaneous injection of 1 mg/kg or 10 mg/kg for 5 days. TGF-ß was given as 1 µg/mouse intracolonically. At 1 mg/kg, the oligonucleotide was as effective as TGF-α in protecting against dextran-sulfate-induced colitis.

Mice were sacrificed on day 6 and colons were subjected to histopathologic evaluation. Until sacrifice, disease activity was monitored by observing mice for weight changes and by observing stools for evidence of colitis. Mice were weighed daily. Stools were observed daily for changes in consistency and for presence of occult or gross bleeding. A scoring system was used to develop a disease activity index by which weight loss, stool consistency and presence of bleeding were graded on a scale of 0 to 3 (0 being

normal and 3 being most severely affected) and an index was calculated. Drug-induced changes in the disease activity index were analyzed statistically. The disease activity index has been shown to correlate extremely well with IBD in general. Results are shown in Figure 16. At 1 mg/kg, the oligonucleotide reduced the disease index by 40%.

Example 21

Antisense oligonucleotide to ICAM-1 increases survival in murine heterotopic heart transplant model

To determine the therapeutic effects of ICAM-1 antisense oligonucleotide in preventing allograft rejection the murine ICAM-1 specific oligonucleotide ISIS 3082 was tested for activity in a murine vascularized heterotopic heart transplant model. Hearts from Balb/c mice were transplanted into the abdominal cavity of C3H mice as primary vascularized grafts essentially as described by Isobe et al., *Circulation* **1991**, 84, 1246-1255. Oligonucleotides were administered by continuous intravenous administration via a 7-day Alzet pump. The mean survival time for untreated mice was 9.2 ± 0.8 days (8, 9, 9, 9, 10, 10 days). Treatment of the mice for 7 days with 5 mg/kg ISIS 3082 increased the mean survival time to 14.3 ± 4.6 days (11, 12, 13, 21 days).

Example 22

Antisense oligonucleotide to ICAM-1 decreases leukocyte migration

Leukocyte infiltration of tissues and organs is a major aspect of the inflammatory process and contributes to tissue damage resulting from inflammation. The effect of ISIS 3082 on leukocyte migration was examined using a mouse model in which carrageenan-soaked sponges were implanted subcutaneously. Carrageenan stimulates leukocyte migration and edema. Effect of oligonucleotide on leukocyte migration in inflammatory exudates is evaluated by quantitation of leukocytes infiltrating the implanted sponges. Following a four hour fast, 40 mice were assigned randomly to eight groups each containing five mice. Each mouse

was anesthetized with Metofane and a polyester sponge impregnated with 1 ml of a 20 mg/ml solution of carrageenan was implanted subcutaneously. Saline was administered intravenously to Group 1 at 10 ml/kg four hours prior to sponge implantation and this served as the vehicle control. Indomethacin (positive control) was administered orally at 3 mg/kg at a volume of 20 ml/kg to Group 2 immediately following surgery, again 6-8 hours later and again at 21 hours post-implantation. ISIS 3082 was administered intravenously at 5 mg/kg to Group 3 four hours prior to sponge implantation. ISIS 3082 was administered intravenously at 5 mg/kg to Group 4 immediately following sponge implantation. ISIS 3082 was administered intravenously at 5 mg/kg to Groups 5, 6, 7 and 8 at 2, 4, 8 and 18 hours following sponge implantation, respectively. Twenty-four hours after implantation, sponges were removed, immersed in EDTA and saline (5 ml) and squeezed dry. Total numbers of leukocytes in sponge exudate mixtures were determined.

The oral administration of indomethacin at 3 mg/kg produced a 79% reduction in mean leukocyte count when compared to the vehicle control group.

A 42% reduction in mean leukocyte count was observed following the administration of ISIS 3082 at 5 mg/kg four hours prior to sponge implantation (Group 3). A 47% reduction in mean leukocyte count was observed following the administration of ISIS 3082 at 5 mg/kg immediately following sponge implantation (Group 4). All animals appeared normal throughout the course of study.

Example 23

Compatibility of antisense oligonucleotide with corneal donor storage media and determination of toxicity

The following studies were performed to determine whether antisense oligonucleotides were toxic to normal ocular tissues. A 20-mer antisense phosphorothioate oligonucleotide (APO) in three different concentrations (40, 200 and 400 µg/ml) was stored in OPTISOL™ corneal donor storage media (Bausch & Lomb) for a total

of 30 days. At day 0, 2, 8 and 30 of incubation, aliquots from each concentration were removed, 2 ml samples were placed in freezer-safe tubes and frozen at -100°C for storage. Samples were thawed and analyzed by capillary gel electrophoresis (CGE). Another 2ml aliquot was obtained for each day for analysis of degradability using by spectrophotometry at 260 nm. The dose response curve in water was linear from 5-200 µg/ml concentrations. The samples containing OPTISOL™ were diluted 1:10 to decrease interference in the spectrophotometer.

No degradation or breakdown components of APO over the 30-day storage period was detected by CGE; however, degradation was observed when analysis was performed by spectrophotometry as indicated as decreased absorbance. Absorbance decreased by 39% after 8 days in the 400 µg/ml samples, 37% in the 200 µg/ml samples and 60% in the 40 µg/ml samples on average. Thus, at the concentrations studied, the APO is stable in OPTISOL™ and does not appear to break down as determined by CGE. Human donor corneas that were unsuitable for transplant were incubated with 3 different concentrations of APO in OPTISOL™ and evaluated after 1, 3 and 8 days using the same criteria applied to corneas for transplant. Corneas were fixed for histologic evaluation by light and electron microscopy. Although all corneas deteriorated over time, low concentrations of APO did not significantly affect either epithelial or endothelial cellular integrity, deturgescence or tissue viability. Thee results for the 1 and 8 day incubations are summarized in Table 6.

Table 6

CORNEAL CHANGES OBSERVED AFTER STORAGE FOR 24 HOURS OR 8 DAYS BY LIGHT MICROSCOPY ANALYSIS

	Edema	Epithelial defect	Inflammation	Absence of polarity
APO (24 h)	1/7	2/7	2/7	3/7
Control(24 h)	0/2	1/2	0/2	0/2
APO (8 days)	3/9	1/9	0/9	7/9
Control(8 days)	1/11	2/11	3/11	9/11

Rabbits were treated with topical doses (200 and 400 µg/ml) of APO for 10 days four times per day. A different concentration was used in each of the two groups. The ocular surface was assessed by clinical examination using the MacDonald-Shadduck toxicology scale. No local toxicity was reported on the MacDonald-Shadduck scale or by light microscopy. The results are shown in Table 7.

Table 7

MACDONALD-SHADDUCK OCULAR IRRITATION SCORES

	Control[1]	APO (40 µg/ml)	APO (400 µg/ml)
Conjunctiva:			
Injection	Normal	Minor[2]	Minor
Chemosis/Swelling	Normal	Minor[3]	Minor
Discharge	None	Minimal	Minimal
Light reflex	Normal	Normal	Slightly sluggish (day 4-8)
Cornea:			
Loss of transparency	None	Minimal (d. 6-7)[4]	Minimal (d. 2-8)
Stromal opacity	None	Minimal (d. 7-8)[5]	Moderate (d. 2-8)[5]
Vascularization	None	None	Minimal[6]
Staining	None	None	None

[1]Vehicle-treated control

[2]Less than 0.5 on a scale of 3.0=minor flushing of palpebral conjunctiva with some perilimbal injection

[3]Less than 0.5 on a scale of 4.0=some swelling without eversion of the lids

[4]Less than 0.5 on a scale of 4.0=some loss of transparency in anterior half of stroma on days 7-8

[5]Minimal>0.5 on a scale of 4.0=>10% area of stromal cloudiness

[6]Moderate 1.0 on a scale of 4.0=<25% area of stromal cloudiness

In addition, serum and aqueous humor were withdrawn and analyzed for the presence of APO to evaluate the ability to penetrate through the corneal tissues. The amount of APO in the serum was less than the limit of detection of the assay method. Significant amounts of APO were found to have penetrated into the aqueous humor, demonstrating the ability of the APO to penetrate through the cornea. After 10 days, the cornea and conjunctiva were studied by light and electron microscopy. By specular microscopy, there were no significant differences between corneas incubated in OPTISOL™ alone or with APO. Light microscopy demonstrated that epithelial polarity and thickness was unaffected by 200 µg/ml and was minimally affected at 400 µg/ml. Scanning electron microscopy (SEM) indicated that storage of corneas up to 8 days did not further increase the time related corneal endothelial degradation.

The experiments described above show that the antisense phosphorothioate oligonucleotides are compatible with corneal storage media, are not toxic to human corneas stored in corneal storage media and are not damaging to normal eye tissue when applied topically.

Example 24

Effect of ISIS 2302 on corneal integrity and tissue viability

Eleven human corneal donor buttons were stored in OPTISOL™ for 8 days and used as the control group. Additional corneal buttons were used for the experimental group and were stored in OPTISOL™ with either 200 µg/ml ISIS 2302 (n=10) or 400 µg/ml ISIS

2302 (n=8). Endothelial cell density was evaluated by specular microscopy. After 8 days, all corneas were prepared for SEM and photographs were taken of endothelial and epithelial surfaces.

Analysis by specular microscopy found that after 2 or 8 days of storage, there was no difference in endothelial cell density among the 3 groups. Both surfaces of the control and experimental groups were analyzed for cellular degradation as well as similarities and differences in their appearance. SEM revealed heavy exfoliation of the epithelial surface of the control group and moderate to heavy pitting and enucleation of the endothelial surface. The corneal buttons exposed to 200 or 400 µg/ml ISIS 2302 were similar in appearance to corneas in the control group. Severe pitting and hollowing of the endothelial surface and shedding of the epithelial surface seem to be consistent in both the control and experimental groups.

Although there were no obvious differences between the experimental and control groups, it should be noted that all corneal buttons were 1-2 days out of the orbit before experimentation began. Furthermore, after eight days in storage, sloughing and loss of the surface cells are to be expected. Thus, ISIS 2302 is not markedly toxic to stored human corneas.

Example 25

Effects of ICAM-1 antisense oligonucleotides (ISIS 9125 and 2105) on allograft rejection

The following study was preformed to determine whether pretreating corneal allografts with the rat ICAM-1 antisense oligonucleotides ISIS 9125 (5'-AGGGCCACTGCTCGTCCACA-3', all 2'-deoxyphosphorothioate) (SEQ ID NO: 86) and ISIS 2105 inhibited corneal allograft rejection. Rejection was induced in rat corneas by removing the corneas from anesthetized donor ACI rats and transplanting them to anesthetized recipient Lewis rats. In this model of corneal transplant rejection, Lewis rat recipients normally produce a rejection reaction within 6-8 days. The cornea transplants were performed after pretreatment of the donor ACI

corneas with either ISIS 9125 or with vehicle (Optisol™) alone. Under surgical anesthesia (ketamine 80 mg/kg, acepromazine 12 mg/kg), a 3 mm section of cornea was removed from one eye of the recipient rat, without damaging internal eye structures. Using the operating microscope, the donor corneal allograft was fitted over the recipient's corneal opening, and 8 to 12 sutures placed aseptically to secure the corneal allograft. Once sutures were in place, the anterior chamber was re-inflated using sterile saline, and tobramycin antibiotic ointment with dexamethasone was applied to the surgical site. The animals were allowed to recover and respiration and behavior were monitored. Some donor corneas were incubated in OPTISOL™ containing 400 µg/ml ISIS 9125 for 24 hours before transplantation.

Rats were examined post-op by slit lamp and rejection was based on the MacDonald-Shadduck scale modified for corneal graft rejection. Rejection criteria included corneal opacity, neovascularization, keratic precipitates and conjunctival inflammation. Following rejection, corneas were harvested for examination under light microscopy (H&E) and SEM. Some corneas were harvested on post-op day 3 for histologic examination. Confocal microscopy was used to document epithelial and endothelial changes *in vivo*.

Corneas transplanted immediately after removal from donor rats rejected an average of 5.94 days (range 4-8 days), while those treated with topical steroid lasted an average of 8.40 days (range 6-11 days). The group whose corneas were incubated in OPTISOL™ for 24 hours rejected an average of 4.80 days (range 3-7 days). Those whose corneas were incubated in OPTISOL™ plus ISIS 9125 for 24 hours rejected an average of 6.33 days (range 6-10 days). By day 3 post-surgery, the ISIS 9125 plus OPTISOL™ group was graded 50% better than the OPTISOL™ alone group for cornea opacity and neovascularization; however, the ISIS 9125 group had more corneal edema than the OPTISOL™ alone group.

A similar procedure was used with ISIS 2105 as the antisense oligonucleotide. The percent of allograft recipients showing no

signs of rejection 3 days post-op in category is shown in Table 8.

Table 8

PERCENT OF ALLOGRAFT RECIPIENTS SHOWING NO SIGNS OF REJECTION 3 DAYS POST-OP IN CATEGORY

Examination item	No pre/post treatment	Post-op steroids alone	24 hr pre-op Optisol storage alone	24 hr pre-op ISIS/Optisol storage
Conjunctival congestion	100	100	100	100
Conjunctival discharge	88	100	100	100
Iris	100	100	100	100
Graft opacity	44	67	50	80
Graft edema	25	33	0	40
Graft neovascularization	0	67	50	100
Graft staining	94	83	100	100
Keratic precips	100	100	100	100

The data show the ability of ISIS 9125 and 2105 to inhibit corneal rejection. Data with steroids, which increased days to rejection by 30%, confirms the validity of the transplant model. ISIS 9125 increased days to rejection by 25% over the 24 hour OPTISOL™ incubation control group. More subtle signs of inflammation were documented *in vivo* by confocal microscopy than could be detected by slit lamp. Although the allograft experiments were conducted with ISIS 9125, the use of other antisense oligonucleotides targeted to cellular adhesion molecules, particularly ICAM-1, VCAM-1 and ELAM-1, for inhibiting corneal allograft rejection is also within the scope of the present invention. The ability of any antisense oligonucleotide targeted to a cell adhesion molecule to inhibit corneal allograft rejection can be easily determined without undue experimentation by using the protocols

described in the present application.

Example 26

Design and screening of duplexed antisense compounds targeting ICAM-1, VCAM-1 or ELAM-1

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target ICAM-1, VCAM-1 or ELAM-1. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide to ICAM-1, VCAM-1 or ELAM-1 as described herein. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini. For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG (SEQ ID NO:87) and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

cgagaggcggacgggaccgTT (SEQ ID NO:88) Antisense Strand
||||||||||||||||||||
TTgctctccgcctgccctggc (SEQ ID NO:89) Complement

In another embodiment, a duplex comprising an antisense strand having the same sequence CGAGAGGCGGACGGGACCG may be prepared with blunt ends (no single stranded overhang) as shown:

cgagaggcggacgggaccg Antisense Strand
||||||||||||||||||||
gctctccgcctgccctggc Complement

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are

annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate ICAM-1, VCAM-1 or ELAM-1 expression according to the protocols described herein.

Example 27

Design of phenotypic assays and *in vivo* studies for the use of HCV inhibitors

Phenotypic assays

Once ICAM-1, VCAM-1 or ELAM-1 inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition.

Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of ICAM-1, VCAM-1 or ELAM-1 in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; Perkin-Elmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs

Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with HCV inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest. Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the ICAM-1, VCAM-1 or ELAM-1 inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

Example 28

dsRNA molecules targeting human ICAM-1

A series of double stranded RNA molecules targeting human ICAM_1 (GenBank Accession Number J03132.1, SEQ ID NO: 90) was designed as shown in Table 9 and prepared according to the methods described above. The oligonucleotide listed in the table is the

antisense strand (shown in the 5' to 3' orientation). The complement of this strand is the sense strand. In all of the RNA compounds described herein, and listed in the various tables, it will be understood that the bases listed as thymidines (T) are, in fact, uridines (U). The corresponding single stranded 5-10-5 MOE gapmer deoxyribonucleotides (all cytidines modified with 5-methyl cytidines) were also tested to compare the activity of the single stranded and double stranded compounds.

RNA inhibition was measured in human T24 cells. The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in real-time quantitative polymerase chain reaction (PCR).

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Table 9: dsRNA molecules targeting human ICAM-1

ISIS #	TARGET SITE	SEQUENCE	%INHIB (DS)	%INHIB (SS)	SEQ ID NO
121725	8	Agaggagctcagcgtcgact	0	0	91
121726	33	Ggctgaggttgcaactctga	0	27	92
121727	256	Ccaggcaggagcaactcctt	0	57	93
121728	321	Ttgaatagcacattggttgg	8	14	94
121729	422	Gcccactggctgccaagagg	18	0	95
121730	571	Tctctcctcaccagcaccgt	0	32	96

121731	674	Aaaggtctggagctggtagg	46	0	97
121732	732	Gcgtgtccacctctaggacc	9	15	98
121733	801	Ccagtgccaggtggacctgg	22	0	99
121734	921	Ccagtattactgcacacgtc	32	72	100
121735	1002	Cctctggcttcgtcagaatc	0	0	101
121736	1121	Ggtggccttcagcaggagct	10	28	102
121737	1221	Catacaggacacgaagctcc	57	25	103
121738	1341	Catcctttagacacttgagc	41	20	104
121739	1421	gctcctggcccgacagaggtt	5	7	105
121740	1501	Gctaccacagtgatgatgac	35	37	106
121741	1622	Ttgtgtgttcggtttcatgg	57	29	107
121742	1633	Ggaggcgtggcttgtgtgtt	2	18	108
121743	1654	Cctgtcccgggataggttca	23	0	109
121744	1666	Cgaggaagaggccctgtccc	51	14	110
121745	1711	Tccactctgttcagtgtggc	23	37	111
121746	1781	Tctgactgaggacaatgccc	61	58	112
121747	1818	Taggtgtgcaggtaccatgg	73	55	113
121748	1924	cctctcatcaggctagactt	50	46	114
121749	1971	Ccagttgtatgtcctcatgg	56	58	115
121750	2012	Gggcctcagcatacccaata	43	37	116
121751	2056	Atgctacacatgtctatgga	63	39	117
121752	2100	Gcccaagctggcatccgtca	29	57	118
121753	2103	Agtgcccaagctggcatccg	25	39	119
121754	2221	Gctccgtgaggccagagacc	7	42	120
121755	2291	Caggcactctcctgcagtgt	3	26	121
121756	2341	Gaaaggcaggttggccaatg	29	32	122
121757	2417	Ggtaatctctgaacctgtga	35	54	123
121758	2531	Gtccagacatgaccgctgag	34	45	124
121759	2619	Ctggagctgcaatagtgcaa	5	22	125
121760	2731	Tacacatacacacacacaca	8	54	126
121761	2831	Gctgaggtgggaggatcact	45	57	127
121762	2871	Ggtgtggtgttgtgagccta	42	67	128
121763	2944	Ctaacacaaaggaagtctgg	48	59	129
121764	2104	Cagtgcccaagctggcatcc	41	60	130

297862	225	Ggtctctatgcccaacaactt	ND	ND	131
297863	282	Cagttcatacaccttccggtt	ND	ND	132
297880	1642	Gtacgtgctgaggcctgcatt	ND	ND	133
348163	1781	Tctgactgaggacaatgccc	ND	ND	134
348164	1781	Tctgactgaggacaatgccc	ND	ND	135
348166	1818	Taggtgtgcaggtaccatgg	ND	ND	136
348167	1818	taggtgtgcaggtaccatgg	ND	ND	137

Example 29

Real-time quantitative PCR analysis of ICAM-1 mRNA levels

Quantitation of ICAM-1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISMJ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the

probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISMJ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 μL PCR cocktail (1x TAQMANJ buffer A, 5.5 mM $MgCl_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLDJ, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μL poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLDJ, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). Probes and primers to human ICAM-1 were designed to hybridize to a human Survivin sequence, using published sequence information (GenBank accession number J03132.1, incorporated herein as SEQ ID NO: 138). For human ICAM-1 the PCR primers were:

forward primer: CATAGAGACCCCGTTGCCTAAA (SEQ ID NO: 139)

reverse primer: TGGCTATCTTCTTGCACATTGC (SEQ ID NO: 140) and the PCR probe was: FAM- CTCCTGCCTGGGAACAACCGGAAX -TAMRA (SEQ ID NO: 141) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

ISIS 121747 (SEQ ID NO: 113) was chosen for further study. A dose response experiment was done in T24 cells using 10, 25, 50, 100 and 200 nM antisense oligonucleotide or corresponding dsRNA.

The results are shown in Table 10.

Table 10

Conc. (nM)	% INHIB (ss)	% INHIB (ds)
10	21	47
25	46	60
50	62	72
100	80	81
200	85	81

Both the ss and ds compounds inhibited expression of ICAM-1 in a dose-dependent manner, with the ds RNA compound exhibiting greater inhibition at all doses tested with the exception of 200 nM.

The dsRNA corresponding to ISIS 2302 (SEQ ID NO: 22) was also tested in T24 cells at 0.5 nM, 5 nM and 50 nM. mRNA levels were measured by Northern blot. Inhibition of mRNA production was 10%, 7% and 20% at these three oligonucleotide concentrations, respectively.

ISIS 121734 (SEQ ID NO:100) (dsRNA) was also tested in T24 cells at 50 nM, 100 nM and 200 nM and inhibited ICAM-1 mRNA expression by 40%, 75% and 58%, respectively.

ISIS 121734 was also tested in a dose response experiment in human T47D breast carcinoma cells. (American Type Culture Collection, Manassas VA). T47D cells were cultured in MEM High glucose media supplemented with 10% FBS (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

Cells were plated at 20,000 cells per well for MCF-7 and T47D cells, and allowed to attach to wells overnight. Plates used were 96 well Costar plate 1603 (black sides, transparent bottom). mRNA levels were determined by real-time quantitative polymerase chain reaction (PCR).

Example 30

Mouse model of allergic inflammation

In the mouse model of allergic inflammation, mice were sensitized and challenged with aerosolized chicken ovalbumin (OVA). Airway responsiveness was assessed by inducing airflow obstruction with a methacholine aerosol using a noninvasive method. This methodology utilized unrestrained conscious mice that are placed into the main chamber of a plthysmograph (Buxco Electronics, Inc., Troy, NY). Pressure differences between this chamber and a reference chamber were used to extrapolate minute volume, breathing frequency and enhanced pause (Penh). Penh is a dimensionless parameter that is a function of total pulmonary airflow in mice (i.e., the sum of the airflow in the upper and lower respiratory tracts) during the respiratory cycle of the animal. The lower the Penh, the greater the airflow. This parameter closely correlates with lung resistance as measured by traditional invasive techniques using ventilated animals (Hamelmann et al., *Proc. Natl. Acad. Sci. U.S.A.* **94**:1350-1355, 1997). Dose-response data were plotted as raw Penh values to increasing concentrations of methacholine. This system was used to test the efficacy of antisense oligonucleotides targeted to ICAM-1.

There are several important features common to human asthma and the mouse model of allergic inflammation. One of these is pulmonary inflammation, in which cytokine expression and Th2 profile is dominant. Another is goblet cell hyperplasia with increased mucus production. Lastly, airway hyperresponsiveness (AHR) occurs resulting in increased sensitivity to cholinergic receptor agonists such as acetylcholine or methacholine. The compositions and methods of the present invention may be used to treat AHR and pulmonary inflammation, particularly asthma.

Ovalbumin-induced allergic inflammation

Balb/c mice (Charles Rivers Laboratory, Taconic Farms, NY), 8-10 weeks of age, weighing about 25 g each, were maintained in micro-isolator cages housed in a specific pathogen-free (SPF)

facility. The sentinel cages within the animal colony surveyed negative for viral antibodies and the presence of known mouse pathogens. Mice were sensitized and challenged with aerosolized chicken OVA. Briefly, 20 μg alum-precipitated OVA was injected intraperitoneally on days 0 and 14. On day 24, 25 and 26, the animals were exposed for 20 minutes to 1.0% OVA (in saline) by nebulization. The challenge was conducted using an ultrasonic nebulizer (PulmoSonic, The DeVilbiss Co., Somerset, PA). Animals were analyzed about 24 hours following the last nebulization using the Buxco electronics Biosystem. Lung function (Penh), lung histology (cell infiltration and mucus production) inflammation (BAL cell type & number) and spleen weight were determined.

Oligonucleotide administration

Antisense oligonucleotides (ASOs) were dissolved in saline and used to intratracheally dose mice every day, four times per day, from days 15-26 of the OVA sensitization and challenge protocol. There were 10 mice/group for Penh and 4 mice/group for broncheoalveolar (BAL) fluid cell type (neutrophil) analysis. The naïve and vehicle groups had 8 mice/group. The only group not sensitized with OVA was naïve mice. Specifically, the mice were anesthetized with isofluorane and placed on a board with the front teeth hung from a line. The nose was covered and the animal's tongue was extended with forceps and 25 μl of various doses of ASO, or an equivalent volume of saline (control) was placed at the back of the tongue until inhaled into the lung. On day 28, lung function measurements (Penh) were taken. The ICAM-1 oligonucleotides used were ISIS 13315 (5'-TGCATCCCCCAGGCCACCAT-3'; SEQ ID NO: 142) and ISIS 17481 (5'-TCCACAGCAGCTTGCACGA-3'; SEQ ID NO: 143). ISIS 13315 contains only phosphorothioate linkages, has 2'-MOE modifications at nucleobases 1-8 and 19, and the cytidines at nucleobases 3, 6, 7 and 8 are 5-methyl cytidines. ISIS 17481 contains only phosphorothioate linkages, has 2'-MOE modifications at nucleobases 13-20, and all cytidines

are 5-methyl cytidines. The treatment groups are shown in Table 11:

Table 11

Groups	Treatment	Dose (mg/kg)	Day 26 sac (BAL)	Day 27 sac (PD)	Day 28 sac (Penh)
1. (N=14)	ISIS 13315	0.03	N=4		N=10
2. (N=14)	ISIS 13315	0.3	N=4		N=10
3. (N=18)	ISIS 13315	3	N=4	N=4	N=10
4. (N=14)	ISIS 17481	0.03	N=4		N=10
5. (N=14)	ISIS 17481	0.3	N=4		N=10
6. (N=18)	ISIS 17481	3	N=4	N=4	N=10
7. (N=18)	vehicle	0	N=4	N=6	N=10
8. (N=18)	Naïve	0	N=4	N=6	N=10

BAL=bronchiolar lavage, PD=pharmacodynamics, Penh=pulmonary airflow

Example 31

Collection of bronchial alveolar lavage (BAL) fluid

Animals were injected with a lethal dose of ketamine, the trachea was exposed and a cannula was inserted and secured by sutures. The lungs were lavaged twice with 0.5 ml aliquots of ice cold PBS with 0.2% FCS. The recovered BAL fluid was centrifuged at 1,000 rpm for 10 min at 4°C, frozen on dry ice and stored at -80°C until used. Luminex was used to measure cytokine levels in BAL fluid and serum.

Example 32

BAL cell counts and differentials

Cytospins of cells recovered from BAL fluid were prepared using a Shandon Cytospin 3 (Shandon Scientific LTD, Cheshire, England). Cell differentials were performed from slides stained with Leukostat (Fisher Scientific, Pittsburgh, PA). Total cell counts were quantified by hemocytometer and, together with the percent type bty differential, were used to calculate specific cell number.

Results of Intratracheal oligonucleotide administration:

The results show a pronounced decrease in Penh after administration of each oligonucleotide which translates to decreased airway hyperresponsiveness in mice after intratracheal administration (Figure 17). As shown in Fig. 22, treatment with ISIS 13315 or ISIS 17481 following allergen (OVA) challenge in the mouse model of asthma reduces the airway response to methacholine (MCH, 100 mg/ml), with ISIS 13315 showing a more pronounced effect. The Penh value in ISIS 13315-treated mice was statistically the same as naïve mice which were not sensitized with the allergen or treated with the antisense oligonucleotide. ISIS 17481 decreased the Penh by one-third at a dose of 3 mg/kg compared to vehicle-treated mice. This shows that ICAM-1 antisense oligonucleotide-treated mice had significantly better airflow, and less inflammation, than mice which were not treated with the antisense oligonucleotide.

The effect of ICAM-1 antisense oligonucleotides on eosinophil and neutrophil recruitment, as measured from BAL fluid, is shown in Figures 18 and 19. Compared to vehicle treated mice, ICAM-1 antisense oligoucleotide-treated mice exhibited reduced number of eosinophils and neutrophils, cells which promote the inflammatory response. The reduction in the

number of eosinophils by ISIS 13315 and ISIS 17481 was similar (Fig. 18). Both antisense oligonucleotides also resulted in decreased numbers of neutrophils in the BAL fluid, with ISIS 13315 exhibiting a greater effect than ISIS 17481 (Fig. 19).

Since increased numbers of eosinophils result from inflammation, this provides further support for the anti-inflammatory properties of ICAM-1 antisense oligonucleotides, particularly in airway and pulmonary inflammatory disorders such as asthma.

In summary, ISIS 13315 and 17481 resulted in an inhibition of airway hypersensitivity, reduced eosinophilia and reduced neutrophilia.

The combined use of antisense oligonucleotide(s) targeted to ICAM-1 with one or more conventional asthma medications including, but not limited to, montelukast sodium (Singulair™), albuterol, beclomethasone dipropionate, triamcinolone acetonide, ipratropium bromide (Atrovent™), flunisolide, fluticasone propionate (Flovent™) and other steroids is also contemplated.

Example 33

Primate Ascaris asthma model

Inhalation of *Ascaris suum* antigen by allergic cynomolgus monkeys exposed to *Ascaris* suum in the wild causes an immediate bronchoconstriction and delayed allergic reaction, including a pulmonary inflammatory infiltrate. This model is described in Turner et al. (*J. Clin. Invest.* **97**:381-387, 1996) and Hart et al., *J. Allergy Clin. Immunol.* **108**:250-257, 2001). Cynomolgus monkeys (n=5 per treatment group) were administered increasing doses of aerosolized methacholine (0.0625, 0.125, 1.0, 4.0, 16.0 and 64.0 mg/ml) to determine a baseline, airway resistance measurements (impedance) and BAL. As described for the ova-induced mouse model, increasing concentrations of methacholine result in increased airway resistance. Monkeys were challenged three and four days after the baseline methacholine dose response at a dose which increased airway resistance by 100-200%. 24 hours later,

another methacholine dose response and BAL were performed at the concentrations listed for the baseline experiment. Experimental animals received 2 mg/kg of aerosolized ICAM-1 antisense oligonucleotide ISIS 10984 (5'-GCCCAAGCTGGCATCCATCA-3'; SEQ ID NO: 144) for four days of treatment: two days and one day prior to antigen challenge and immediately before the two antigen challenges. Monkeys were anesthetized, intubated and maintained on a ventilator for each procedure. ISIS 10984 contains all phosphorothioate linkages.

The minimum response was the 16 mg/ml dose of methacholine for a good responder, and 4 mg/ml for an excellent responder. Thr results are shown in Fig. 20. In the figure, methacholine doses increase 4 fold; every 10 min. another dose of methacholine is aerosolized; and minimal is defined as that response tht elicits at least a 40-50% increase in airway resistance during baseline conditions (pre-antigen). The results show that pretreatment of aerosolized ICAM-1 antisense oligonucleotide (ISIS 10984) significantly reduces airway impedance (resistance) in cynomolgus monkeys at all methacholine concentratons tested. The effect of pretreatment with ISIS 10984 on bronchial cell influx in BAL was also determined 24 hours after two consecutive day antigen challenge in Ascaris sensitive cynomolgus monkeys. Pretreatment had no effect on polymorphonuclear cells (PMNs), macrophages or eosinophils following *Ascaris suum* challenge.

Two other oligonucleotides suitable for use in the present invention are ISIS 15839 (5'-GCCCAAGCTGGCATCCGTCA-3'; SEQ ID NO: 145; all phosphorothioate linkages; all cytidines are 5-methyl cytidines, nucleobases 13-20 comprise 2'-MOE modifications) and ISIS 15537 (5'-TCTGAGTAGCAGAGGAGCTC-3'; SEQ ID NO: 146; all phosphorothioate linkages; all cytidines are 5-methyl cytidines; all nucleobases comprise 2'-MOE modifications).